MEDICAL LIBRARY

OCT 9 1948

Vol. 3. No. 3. SEPTEMBER 1948

BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY

EDITED FOR

THE BRITISH PHARMACOLOGICAL SOCIETY

BY

J. H. BURN F. HAWKING

A. D. MACDONALD N. MUTCH C. M. SCOTT

J. H. GADDUM (Chairman) H. R. ING (Secretary)

In co-operation with the EDITOR of the British Medical Journal

LONDON

BRITISH MEDICAL ASSOCIATION

TAVISTOCK SQUARE, W.C.1

YEARLY SUBSCRIPTION (4 NUMBERS) 25s. U.S.A. $6.00 SINGLE NUMBER 7s. 6d.

NOTICE TO CONTRIBUTORS

The JOURNAL, which appears quarterly, publishes original work in all branches of pharmacology and experimental chemotherapy, including the biochemical and pathological aspects of the subject. Provision is also made for short communications and for brief notes on technique. Papers submitted for publication and all editorial communications should be addressed to Dr. H. R. Ing, Department of Pharmacology, Oxford.

Papers submitted to the JOURNAL are accepted on the understanding that they have not been and will not be published in whole or in part in any other journal; they are subject to editorial revision.

MSS. and illustrations should bear the name and full address of the author to whom proofs are to be sent. Papers should be in double-spaced typing on one side only of quarto or foolscap sheets, with a margin of not less than 1½ in. Papers must be as concise as possible, and only recognized abbreviations should be used.

In stating the object of the research the author should refer to previous work only if it has a direct bearing on the subject of the paper; as a rule a complete historical review is unnecessary. A paper should conclude with a brief but self-explanatory summary giving in numbered paragraphs the results and conclusions described in the paper; it should not exceed 1-20th of the length of the paper.

TABLES.—Tables are to be typed on separate numbered sheets and referred to in the text by Roman numerals. Tables should be comprehensible without reference to the text.

ILLUSTRATIONS.—Illustrations are to be referred to in the text by Arabic numerals. Legends or captions are to be typed on separate sheets and numbered correspondingly; they should render the illustrations comprehensible without reference to the text. Line drawings and graphs, which should be about twice their intended final size, are to be carefully drawn in Indian ink on tracing linen, or Bristol board, or stout smooth white paper; blue-lined graph paper of good quality may be used for graphs. Lettering of drawings and graphs must be lightly written in pencil. Lettering on Kymograph records should be large enough to be legible on photographic reproduction. When half-tone reproduction of *x*-ray or other illustrations is required the author is advised to send the original film unless he wishes to bring out special points in a print of his own choice. Photographs and photomicrographs should be printed on glossy paper, should be larger than the size desired for reproduction, and, if transmitted through the post in a tube, should be rolled with the picture outside.

REFERENCES.—References should be arranged according to the Harvard system. In the text the year of publication must follow the author's name, more than one paper in any one year being indicated by a small letter (a,b,c) after the date. At the end of the paper references should be arranged in the alphabetical order of the authors' names and should include: author's name, followed by initials; year of publication, in parentheses; title of publication, underlined, and abbreviated in accordance with the *World List of Scientific Periodicals*; volume number in Arabic numerals underlined with a wavy line to indicate bold type; the number of the first page in Arabic numerals, thus: Barger, G., and Dale, H. H. (1910–11). *J. Physiol.*, 41, 19.

The BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY should be referred to in the list of references as *Brit. J. Pharmacol.* When reference is made to a book the author's name and the date should be followed by: title, underlined; edition, volume number (vol.), and page (p. or pp.); town of origin; and the publisher, thus: Clark, A. J. (1940). *Applied Pharmacology*, 7th ed., p. 378. London: Churchill.

For further information on the preparation of MSS. for publication authors are recommended to study the "Suggestions to Authors" published by the Editorial Board of the *Journal of Physiology* (1945), **104**, 221.

PROOFS.—Contributors will receive one proof in page, but it will be assumed that all but verbal corrections have been made in the original MS.; an allowance at the rate of ten shillings per sheet of sixteen pages is made for alterations in the proof (printer's errors excepted), and contributors will be charged for any excess.

REPRINTS.—Fifty reprints of an article will, if desired, be given to an author or divided among the authors. A limited number of additional reprints at cost price can be supplied if application is made when the proofs are returned, and an estimate of costs can be obtained from the Publishing Manager, British Medical Association.

COPYRIGHT.—Papers which have been published become the property of the JOURNAL and permission to republish must be obtained from the Editors.

Brit. J. Pharmacol. (1948), **3**, 181.

NITROAKRIDIN 3582: A COMPOUND POSSESSING CHEMOTHERAPEUTIC ACTIVITY AGAINST THE VIRUSES OF PSITTACOSIS AND LYMPHOGRANULOMA VENEREUM

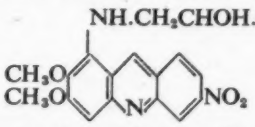
BY

E. WESTON HURST

From Imperial Chemical Industries Limited, Biological Laboratories, Hexagon House, Manchester, 9

(Received November 5, 1947)

"Nitroakridin 3582" (Höchst) or 2,3-dimethoxy-6-nitro-9(3′-diethylamino-2′-hydroxypropyl)amino-acridine dihydrochloride



was developed by the Germans, who claimed that it possessed activity against rickettsiae. Smadel, Snyder, Hamilton, Fox, and Jackson (1946) found it to exert a beneficial effect on mice and embryonated eggs experimentally infected with murine or epidemic typhus, tsutsugamushi disease or Rocky Mountain spotted fever. Green, Rasmussen, and Smadel (1946) observed a chemotherapeutic effect of the compound in embryonated eggs infected with the Lee strain of influenza B virus. In most of their experiments they mixed the compound with virus and inoculated the mixture into the allantoic sac of 11-day embryos. In two experiments nitroakridin was injected an hour before virus. After 2–5 days they estimated virus in the allantoic fluid by means of the haemagglutination technique. Only 3 of 107 eggs receiving 1–10 M.I.D. of virus together with 0.5 mg. nitroakridin agglutinated red cells, and then to a lower titre than did infected control eggs. Against 100 or more M.I.D. of virus the compound exerted a less striking effect. The addition of nitroakridin to known positive allantoic fluids did not influence their titres; nor did the drug inactivate virus at room temperature in 15 minutes, about 5 minutes longer than the time occupied in inoculating eggs after preparation of a mixture.

This claim of therapeutic activity against one of the influenza viruses seemed sufficiently important to warrant investigation of the action of the compound against other viruses. My colleague, Dr. P. Gaubert, very kindly prepared a sample of nitroakridin, with which we obtained no favourable effect on influenza in mice or on infections caused by four neurotropic viruses. Against psittacosis and lymphogranuloma venereum, however, the compound showed moderate activity.

EXPERIMENTAL

In suitable chronic toxicity tests the maximal tolerated dose of nitroakridin in mice weighing 20 g. was 0.25 mg. daily, given intraperitoneally as a single dose dissolved in 0.5 c.c. sterile distilled water. In many experiments the animals were infected with a virus two hours after the second dose; variations from this procedure are indicated in the appropriate places. Dosing continued for a variable period according to the virus in use and the exact object of the particular experiment. Most six-day-old chick-embryos tolerated a single dose of 0.5 mg. injected into the yolk-sac two hours after virus had been given by the same route.

In comparative tests with other drugs we gave sulphonamides orally twice daily in doses of 5 mg. per 20 g., penicillin (96 per cent penicillin-II) by intraperitoneal injection of 500 units four times daily at 9.0 a.m., 12.45, 5.0 and 9.30 p.m. During the night, animals receiving penicillin were offered the drug dissolved in sterile distilled water from sterilized drinking bottles.

RESULTS

(a) Influenza

Table I sets forth the scores of the pulmonary lesions in mice infected intranasally with the PR-8 strain of influenza virus A and killed on the 7th day. The results in mice treated with nitroakridin do not suggest a therapeutic effect of the compound.

In the haemagglutination test nitroakridin did not inhibit agglutination of fowl erythrocytes by influenza virus; on the contrary, the stronger solutions themselves produced strong agglutination.

TABLE I

EFFECT OF TREATMENT WITH NITROAKRIDIN ON THE PULMONARY LESIONS OF MICE INFECTED INTRANASALLY WITH INFLUENZA VIRUS

5 = death with specific lesions on or before the 7th day. 4–0 = extent of pulmonary lesions recorded according to conventional practice.

Exp.	Number of doses of drug	Dilution of virus	Pulmonary lesions	
			Treated mice	Untreated mice
1	7	10^{-5} 10^{-6} 10^{-7}	4, 4, 3, 3, 3, 3, 3, 1, 0, 0 = 24 4, 3, 3, 2, 1, 1, 1, 1, 0, 0 = 16 1, 1, 1, 1, 0, 0, 0, 0, 0, 0 = 4	5, 5, 4, 4, 4, 3, 3, 3, 2, 1 = 34 4, 3, 3, 2, 2, 1, 1, 1, 0, 0 = 17 1, 1, 1, 1, 1, 0, 0, 0, 0, 0 = 5
2	7	10^{-4} 10^{-5} 10^{-6}	5, 5, 5, 5, 5, 5, 3, 2, 2, 0 = 37 3, 3, 3, 2, 2, 2, 1, 1, 1, 0 = 18 4, 3, 3, 2, 2, 1, 1, 1, 0, 0 = 17	5, 5, 5, 5, 5, 5, 4, 4, 4, 3, 2 = 42 5, 5, 4, 3, 2, 2, 2, 1, 1, 0 = 25 3, 2, 2, 2, 2, 2, 2, 1, 0, 0 = 16
3	8	10^{-4} 10^{-5} 10^{-6}	5, 5, 5, 5, 5, 5, 5, 5, 4, 4 = 48 5, 5, 4, 4, 4, 3, 3, 2, 0, 0 = 30 3, 2, 2, 2, 2, 2, 1, 1, 1, 0 = 16	5, 5, 5, 5, 5, 5, 4, 4, 4, 4, 3 = 44 5, 5, 4, 4, 3, 3, 3, 2, 2, 0 = 31 4, 3, 3, 3, 2, 2, 2, 1, 0, 0 = 20

(b) Equine encephalomyelitis, louping-ill, St. Louis encephalitis and rabies

Table II shows the mortality in groups of 30 mice treated or untreated with nitroakridin and infected with one or other neurotropic virus. In Eastern equine encephalomyelitis the compound apparently prolonged the mean period of survival slightly; clinically we noticed that several animals lingered on in a moribund state, an experience unusual with this infection. In louping-ill the compound produced a heavier mortality, which statistically was almost significant, and the average period of survival was shorter than in controls. With the virus of St. Louis encephalitis deaths were very much more numerous in treated animals, though the average period of survival appeared to be prolonged. In rabies the death-rate was slightly but not significantly greater than in control animals, the period of survival being unaltered.

None of these results suggests that nitroakridin may be a useful therapeutic agent for the diseases in question.

(c) Psittacosis

From the beginning it was clear that nitroakridin had a clear-cut beneficial effect on psittacosis. This effect was apparent not only from statistical consideration of the completed experiments; the

TABLE II

EFFECT OF TREATMENT WITH NITROAKRIDIN OF MICE INFECTED WITH NEUROTROPIC VIRUSES

Virus	Dose	Route of inoculation	Mortality in groups of 30 mice				
			Treated mice			Untreated mice	
			Number of doses of drug	Deaths	Mean period of survival in days*	Deaths	Mean period of survival in days*
Equine encephalomyelitis (Eastern strain)	1000 cerebral LD50	intramuscular	8	10	6.2	9	5.0
Louping-ill	1000 cerebral LD50	intramuscular	8	16	12.9	9	14.5
St. Louis encephalitis	1 cerebral LD50	intracerebral	14	25	18.0	8	14.9
Rabies (virus-fixe) ..	1000 cerebral LD50	intramuscular	14	25	15.1	19	14.9

* From time of inoculating virus.

TABLE III

EFFECT OF TREATMENT WITH NITROAKRIDIN OF MICE INFECTED WITH PSITTACOSIS

Exp.	Dilution of virus	Route of inoculation of virus	Day exp. ended	Mortality in groups of 30 mice					
				Treated mice				Untreated mice	
				Number of doses of drug	Time of first dose of drug	Deaths	Mean period of survival in days*	Deaths	Mean period of survival in days*
1	$10^{-6.5}$	intraperitoneal	22	8	26 hr. before virus	15	12.0	28	8.6
2	$10^{-7.0}$	intraperitoneal	26	8	26 hr. before virus	11	12.0	27	9.3
		,,	26	14	26 hr. ,, ,,	2	13.0	28	9.5
3	$10^{-7.0}$	intranasal	28	21	26 hr. before virus	20	15.2	25	15.2
		intraperitoneal	28	15	26 hr. ,, ,,	16	11.9	29	8.6
		,,	28	12	48 hr. after virus	22	11.9		
		,,	28	10	96 hr. ,, ,,	24	9.5		
4	$10^{-7.5}$ = 5 LD50	intraperitoneal	42	13	4 hr. after virus	13	14.3	28	7.1
	$10^{-7.0}$ = 10 LD50	,,	42	13	4 hr. ,, ,,	13	21.4	28	7.2
	$10^{-5.0}$ = 1000 LD50	,,	42	13	4 hr. ,, ,,	16	12.2		
	$10^{-7.0}$ = 10 LD50	,,	42	13	52 hr. ,, ,,	23	13.5		
5	$10^{-7.5}$ = 5 LD50	intraperitoneal	36	13	4 hr. after virus	11	19.1	27	15.4
	$10^{-7.0}$ = 10 LD50	,,	36	13	4 hr. ,, ,,	8	21.0	29	15.4
	$10^{-5.0}$ = 1000 LD50	,,	36	13	4 hr. ,, ,,	19	17.9		
	$10^{-7.0}$ = 10 LD50	,,	36	13	52 hr. ,, ,,	12	19.4		
6	$10^{-7.0}$	intraperitoneal	40	14	26 hr. before virus	26	19.7	26	11.5
	$10^{-5.0}$	,,	40	14	26 hr. ,, ,,	19	11.3	30	7.9
	$10^{-4.0}$	,,	40	14	26 hr. ,, ,,	27	10.3	26	7.5

* From time of inoculating virus.

casual visitor to the isolation-unit, at a time when untreated animals were dying, might see one cage of extremely sick (control) mice, and another containing more numerous and more active animals of which few or none were obviously ailing. Table III illustrates a number of points regarding this therapeutic action.

Experiment 1 demonstrated the decreased mortality and the longer survival time of treated as compared with untreated mice inoculated intraperitoneally.

Experiment 2 showed that while comparable control groups gave remarkably uniform results, extension of the period of treatment from 6 to 12 days after infection (8 to 14 doses including those before infection) greatly enhanced the therapeutic effect.

Experiment 3 failed to demonstrate an effect of similar magnitude when virus was instilled intranasally. Whereas when virus was injected intraperitoneally nitroakridin reduced mortality considerably and increased mean-survival-time, more prolonged administration of the drug had less effect upon the number of deaths and none on the mean-survival-time of mice inoculated intranasally. Even so, a definite effect was apparent in additional groups of mice treated exactly as the others, except that they were killed on the 8th day after intranasal inoculation and the focal lesions in the lungs counted. The foci in the control (untreated) group of 30 animals numbered 68, while those in the treated group of similar size numbered only 28. The other point established by Experiment 3 is that after intraperitoneal injection of virus delays in beginning treatment, of 48 and 96 hours respectively, resulted in a reduced favourable effect ; even with the longer period of delay, however, the tendency was towards more frequent or more prolonged survival than in control mice. Confirmatory data on this point were obtained in Experiments 4 and 5. The partial benefit conferred by delayed treatment dispelled the suspicion that the greater efficacy of nitroakridin against intraperitoneally administered virus might be attributed to direct inactivation of the latter by drug still present in the abdominal cavity at the time of injection. Further evidence pointing to this conclusion will be

considered later. In *in vitro* experiments designed to elucidate its mode of action we found that concentrations of the drug of from 1 : 10,000 to 1 : 1,000 did not inactivate the virus fully within one hour ; the mixtures still killed most of the mice inoculated, though with the 1 : 1,000 concentration the incubation period of the disease was prolonged by one or two days.

In Experiments 4–6 the period of observation of the animals was extended and the effect of various doses of virus studied. If a large dose of virus be given (10^{-4}), nitroakridin is apparently capable only of increasing survival-time without preventing ultimate death. With a dilution of 10^{-5}, and often 10^{-7}, a considerable therapeutic effect is seen. When the dose of virus is very small, however, there is a tendency towards rather poorer therapeutic results, particularly evident in Experiment 6. In that experiment, at the time when administration of the drug ceased most of the controls were dead while nearly all the treated mice were alive and apparently quite well. About a week later many began to sicken, and the final mortality in this group was the same as among the controls. This reactivation of the disease, which we have seen on several occasions, did not come wholly as a surprise, because we had previously discovered that treatment with nitroakridin does not eradicate virus from the mice ; apparently healthy animals prevented from dying by such treatment carry fully virulent virus in a greatly enlarged spleen for at least a month. The fact that this reactivation tends to occur more often after small than after larger doses of virus would seem to merit further study ; it recalls the recent observation of Peterson and Fox (1947) in tsutsugamushi disease treated with methylene blue that treatment may safely be discontinued sooner after a massive infecting dose than after a smaller one.

Table IV shows the effect of varying the dosage of nitroakridin ; the best results appear to follow a dose of 0.25 mg. given once daily.

In Table V the effect of giving virus and drug by different routes is shown. It appears that intravenous administration of the drug is more effective against intraperitoneally inoculated virus than is intraperitoneal medication. Medication by the latter route, however, clearly has an effect on both intraperitoneal and intravenous infections. These observations furnish additional support for the conclusions reached previously, viz., that the action of nitroakridin is not purely a local one in the peritoneal cavity.

TABLE IV

CHEMOTHERAPY OF PSITTACOSIS

The effect of various doses of nitroakridin
All treatments began 4 hours after intraperitoneal injection of virus.

Dosage of Nitroakridin	Number of doses	Mortality in groups of 30 mice	
		Deaths	Mean period of survival in days
None	—	28	7.2
0.25 mg. in 0.5 c.c. water once daily	13	13	21.4
0.125 mg. in 0.25 c.c. water twice daily ..	26	21	10.8
0.1 mg. in 0.25 c.c. water once daily	13	26	10.2
0.5 mg. in 1.0 c.c. water once every three days	6	21	11.5

TABLE V

CHEMOTHERAPY OF PSITTACOSIS

The effect of varying the route of administration of virus and of nitroakridin
Infecting dilution of virus 10^{-6}. All treatments began 4 hours after injection of virus and continued daily for 16 doses. Animals observed for 37 days

Inoculation of virus	Injection of drug	Mortality in groups of 30 mice			
		Treated mice		Untreated mice	
		Deaths	Mean period of survival in days*	Deaths	Mean period of survival in days*
intraperitoneal	intraperitoneal	20	12.9	28	11.8
intraperitoneal	intravenous	8	17.6	—	—
intravenous	intraperitoneal	17	17.6	23	13.6

* From time of inoculating virus

Finally we made several comparisons of the activity of nitroakridin and other chemotherapeutic substances. Table VI presents the results of one such experiment. With our strain of psittacosis virus sulphadiazine and sulphamezathine consistently failed to prevent death and usually prolonged but slightly the mean period of survival ; sometimes sulphadiazine appeared to be the more active, at other times sulphamezathine. In the experiment shown in Table VI, nitroakridin, while failing to influence the final mortality, increased considerably the mean period of survival. It was, however, not nearly as efficacious as penicillin, though even after 18 days of therapy with this substance virus was not eradicated ; many of the mice, apparently well throughout the period of therapy, began to sicken and die five to ten days after treatment was discontinued.

TABLE VI

CHEMOTHERAPY OF PSITTACOSIS

Comparison of nitroakridin with other chemotherapeutic substances
Dosing began one hour after intraperitoneal injection of virus and continued for 18 days. Animals were observed for 35 days.

	Mortality in groups of 30 mice	
	Deaths	Mean period of survival in days
Controls	26	8.3
Sulphadiazine (5 mg./20 g. twice daily, orally)	28	9.2
Sulphamezathine (5 mg./20 g. twice daily, orally)	26	8.7
Nitroakridin (0.25 mg./20 g. once daily, i.p.)	29	12.4
Penicillin (500 units/mouse 4 times daily, i.p.)	19	23.8

*(d) Lymphogranuloma venereum**

Our observations with this virus supply the data for Table VII. Virus inoculated into the yolk-sac killed all untreated embryos within 8 days, and most by the 6th day. A dose of 0.5 mg. nitroakridin injected into the yolk-sac two hours after virus killed a number of embryos within the first 24 hours. Of the remainder a few survived to the 20th day, while most of those dying did so at a later stage than the controls. A dose of 0.25 mg. per egg resulted in no toxic effects but all embryos succumbed to lymphogranuloma, albeit more slowly than the controls. Thus, against lymphogranuloma in chick-embryos nitroakridin exhibits moderate chemotherapeutic activity.

* Alternatively known as lymphogranuloma inguinale and climatic bubo.

COMMENT

It is well known that the virus of lymphogranuloma venereum is susceptible to chemotherapeutic attack by many sulphonamides. MacCallum and Findlay (1938), Findlay (1940), Jones, Rake, and McKee (1941), Rodaniche (1942), Felton, Hebb, and Oliphant (1943), van den Ende and Lush (1943) and others have dealt with various aspects of their action against the experimental disease in mice, and Meiklejohn, Wagner, and Beveridge (1946) in the chick-embryo. The last authors also showed that penicillin inhibited growth of this virus in the yolk-sac.

Reports of the activity of the commoner antibacterial agents against the psittacosis virus have not been unanimous. Rudd and Burnet (1941) failed to demonstrate in mice a therapeutic action of several sulphonamides, though Wiseman, Meiklejohn, Lackman, Wagner, and Beveridge (1946) and Early and Morgan (1946b) found sulphadiazine to be effective in mice, and Meiklejohn, Wagner, and Beveridge (1946) and Early and Morgan (1946a) in eggs. Heilman and Herrell (1944), Bedson and May (1945), Meiklejohn *et al.* (1946), Wiseman *et al.* (1946), and Early and Morgan (1946a and b), using mice, developing eggs, or tissue-cultures, have all observed activity on the part of penicillin. The last-named authors noted that the antibiotic was less effective against intranasal than against intravenous infection in mice, and ineffective against intracerebral inoculation; oral sulphadiazine was apparently less effective against intraperitoneal than against intravenous or respiratory infection.

Mauer (1938) reported that trypaflavin exerted a beneficial action in mice infected with psittacosis. With this exception the present appears to be the

TABLE VII

EFFECT OF NITROAKRIDIN ON CHICK-EMBRYOS INFECTED WITH LYMPHOGRANULOMA VENEREUM

Exp.	Dose per egg mg.	Results in groups of 15 eggs							
		Treated				Untreated			
		Non-specific deaths	Specific deaths	Survived	Mean period of survival in days†	Non-specific deaths	Specific deaths	Survived	Mean period of survival in days†
1	0.50	4	8	3	9.9	0	15	0	6.5
2	0.50	6	7	2	8.0	0	15	0	5.4
3	0.25	0	15	0	7.6	0	15	0	4.0

† Of embryos dying specifically.

first record of the activity of an acridine compound against viruses of the psittacosis—lymphogranuloma group.

We have shown that nitroakridin 3582 (Höchst) is moderately active against the viruses of psittacosis in mice and lymphogranuloma venereum in developing chick-embryos. Against our strain of psittacosis virus it appears more active than sulphadiazine or sulphamezathine (which indeed have only slight therapeutic properties) and much less active than penicillin. It is less active against intranasal than against intraperitoneal or intravenous infections.

Nearly all authors, including Rodaniche (1943), agree that treatment with current antibacterial agents does not sterilize infections with these viruses, and that despite clinical improvement or survival the animals continue to harbour fully virulent virus. In our experiments we have confirmed this observation for psittacosis and nitroakridin ; we have further observed that after treatment for as long as 18 days with nitroakridin or penicillin apparently healthy survivors are apt, a week or more later, to develop symptoms of psittacosis and die.

Summary

Nitroakridin 3582 (Höchst) possesses moderate therapeutic activity against the viruses of psittacosis and lymphogranuloma venereum. It is more active than sulphadiazine and sulphamezathine but less active than penicillin. A week or more after the termination of a prolonged course of either nitroakridin or penicillin mice which have apparently been enabled successfully to withstand infection with psittacosis virus frequently develop symptoms and die. This observation accords with the general experience that the common antibacterial agents, while suppressing infections with viruses of the psittacosis–lymphogranuloma group, do not eradicate virus, which may still persist in a fully virulent state.

I am indebted to Prof. S. P. Bedson for the strain of psittacosis virus used in this work (M.O.H.154).

References

Bedson, S. P., and May, H. B. (1945). *Lancet*, **2**, 394.
Early, R. L., and Morgan, H. R. (1946a). *J. Immunol.*, **53**, 151.
Early, R. L., and Morgan, H. R. (1946b). *J. Immunol.*, **53**, 251.
Felton, L. D., Hebb, A., and Oliphant, J. W. (1943). *Chem. Abstr.* **37**, 1197.
Findlay, G. M. (1940). *Lancet*, **2**, 682.
Green, R. H., Rasmussen, A. F., and Smadel, J. E. (1946). *Pub. Hlth Rep. U.S. Pub. Hlth Service*, **61**, 1401.
Heilman, F. R., and Herrell, W. E. (1944). *Proc. Mayo Clin.*, **19**, 57.
Jones, H. P., Rake, G., and McKee, C. M. (1941). *Proc. Soc. exp. Biol. Med.*, **48**, 318.
MacCallum, F. O., and Findlay, G. M. (1938). *Lancet*, **2**, 136.
Mauer, G. (1938). *Zbl. Bakt.* (1 Abt. Orig.), **142**, 279.
Meiklejohn, G., Wagner, J. C., and Beveridge, G. W. (1946). *J. Immunol.*, **54**, 1.
Peterson, O. L., and Fox, J. P. (1947). *J. exp. Med.*, **85**, 543.
Rodaniche, E. C. (1942). *J. infect. Dis.*, **70**, 58.
Rodaniche, E. C. (1943). *J. infect. Dis.*, **73**, 173.
Rudd, G. V., and Burnet, F. M. (1941). *Aust. J. exp. Biol. med. Sci.*, **19**, 33.
Smadel, J. E., Snyder, J. C., Hamilton, H. L., Fox, J. P., and Jackson, E. B. (1946). *Fed. Proc.*, **5**, 254.
van den Ende, M., and Lush, D. (1943). *J. Path. Bact.*, **55**, 81.
Wiseman, R. W., Meiklejohn, G., Lackman, D. B., Wagner, J. C., and Beveridge, G. W. (1946). *J. Immunol.*, **54**, 9.

Brit. J. Pharmacol. (1948), **3**, 187.

THE TOXIC PRINCIPLE OF *COURBONIA VIRGATA*: ITS ISOLATION AND IDENTIFICATION AS A TETRAMETHYLAMMONIUM SALT

BY

A. J. HENRY

From the Wellcome Chemical Laboratories, Sudan Medical Service, Khartoum

(Received November 6, 1947)

A tuberous root submitted for examination from the Southern Sudan as having been concerned in the death of two women and since identified at Kew as *Courbonia virgata A. Brongn.*, a member of the *Capparidaceae*, has been found to contain a toxic principle of an unusual nature.

The highly toxic nature of the root was immediately confirmed by oral administration of aqueous extracts to rabbits, but preliminary examination for alkaloids yielded negative results and no evidence could be found for the presence of a glucoside. Purification of the aqueous extract by treatment with basic lead acetate was found, by toxicity tests, to leave the toxic principle in solution, and it was observed that the purified solution, after removal of excess lead with hydrogen sulphide, gave an immediate precipitate of well-formed dark green crystals with a metallic lustre on addition of Wagner's reagent (iodine in potassium iodide). This crystalline precipitate contained free iodine, and was evidently the periodide of a base. On treatment with hot water it slowly dissolved with liberation of free iodine, which could ultimately be driven off completely. After repeated evaporation of the resulting solution to dryness, baking at 100° C. and taking up with water, followed by fractional crystallization, the iodide of the base was finally obtained in pure form as colourless, well-formed crystals. Injection of an aqueous solution of this salt into rabbits and mice showed it to be highly toxic and to produce symptoms closely similar to those produced by the aqueous infusion of the root which had been originally tested. The above treatment of the material had therefore successfully isolated, in the form of its iodide, the main toxic principle present in the root. Analysis of the periodide, re-formed from the purified iodide, has shown it to approximate to the formula BI_3.

In conformity with the results obtained on the original root extract, it was shown that the base is not precipitated on alkalinization of a solution of the iodide with ammonia or caustic soda, and is not extracted from alkaline solution by the usual organic solvents; this indicated a base of an unusual type, most probably containing a quaternary nitrogen atom. Moreover the action of silver hydroxide on the iodide produced a strongly alkaline solution the conductivity of which was as high as that of a solution of caustic soda of equivalent concentration, and which, on evaporation to dryness, yielded a very hygroscopic solid.

The iodide is very stable to heat. Up to about 400° C. there was little sign of melting or sublimation, but on further heating an oily sublimate was produced. An aqueous solution of the iodide was neutral, optically inactive, non-fluorescent, odourless, and appeared to be quite stable. Analysis of the iodide was not possible here owing to lack of facilities for carrying out combustions. Even a satisfactory estimation of nitrogen could not be carried out, as the compound either shows an extraordinary resistance in the Kjeldahl analysis (even with a mercury-selenium catalyst) or loses nitrogen during the digestion. In consequence, a specimen of the salt was submitted to Dr. Harold King of the National Institute for Medical Research, Hampstead, and I am deeply indebted to him for its identification as tetramethylammonium iodide (Found: C, 24.4; H, 6.0; N, 6.8; I, 62.6. Calc. for $C_4H_{12}NI$: C, 23.9; H, 6.0; N, 7.0; I, 63.1 per cent). Its picrate melted with decomposition at 315° C. as did an authentic specimen, and a mixture of the two showed an identical behaviour.

This is the first occasion on which a salt of tetramethylammonium hydroxide has been found in the vegetable kingdom. Ackermann, Holtz, and

Reinwein (1923) found it in the animal kingdom in a sea-anemone (*Actinia equina*) and gave it the name tetramine.

As a qualitative test for the toxic principle of *C. virgata* (tetramine) the crystalline precipitate which it produces with Wagner's reagent can be used. From warm, dilute solutions, either acid or neutral, the periodide rapidly separates as well-shaped rhombic crystals which are readily recognizable under the microscope. By this means it has been shown that tetramine also occurs in the thick, scaly shoots of the superstructure of the plant, and in the leaves of the subsidiary shoots. Its presence in the shoots and leaves of the specimen sent to Kew was confirmed in the same way. Another specimen of leaves submitted from the South for identification purposes, and reputed to be of the same species as that involved in the fatalities, was identified at Kew as being *Osyris compressa* (Berg.) A. DC., " . . . absurdly like *C. virgata* in the sterile state and could very easily be confused with it in the field." A purified extract of these leaves failed to give crystals of any sort with Wagner's reagent. From the incomplete data at present available it is estimated that the fresh root of *C. virgata* contains about 0.2 per cent of the toxic base, and that about 0.25 g. of the base, taken orally, has proved a lethal dose for adult human beings, death occurring within an hour. The victims " . . . did not vomit but appeared drunk. They threw their arms about, babbled incoherently, and were difficult to control." Subcutaneous injection into mice showed the lethal dose of the iodide, by this route, to be 0.5–1.0 mg. per 25 g. mouse, the observed symptoms being convulsive spasms, collapse, and death within 30 minutes. Intravenous injection of 8 mg. of the iodide into a rabbit caused death within two minutes. With rabbits some contraction of the pupils has been observed prior to death. Daily sub-lethal doses (0.25 mg.), during which 5 mg. were administered subcutaneously, failed to confer any immunity on a mouse or to reveal any cumulative action of the poison, as, at the end of the series of injections, the mouse was badly shocked by injection of 1 mg. and four days later was killed within two minutes by injection of 2 mg.

From these results it is evident that *C. virgata* is to be classified as a relatively highly toxic plant, and from the toxicologist's point of view failure of the toxic principle to be extracted from alkaline solution by organic solvents is important. Unless its presence is suspected, and its properties known, it might easily be overlooked. After addition of 0.02 g. of the iodide to 200 g. of macerated liver the normal Stas-Otto procedure for recovery of alkaloids from such material yielded an aqueous solution of the toxic principle which, after further purification with basic lead acetate, gave an excellent positive result with Wagner's reagent. The liver of the rabbit killed by intravenous injection of 8 mg. of the iodide failed to give a definite positive result on application of this procedure. The toxic properties of *C. virgata* appear to be well known to the natives of the areas where the plant occurs (e.g., it is said to be used for poisoning hyenas), so that its toxicological aspect, about which nothing was previously known, is likely to prove important in such regions. The plant is known to occur in northern Uganda, north-eastern Kenya, and parts of French Equatorial Africa, as well as in the Southern Sudan.

A solution of tetramine iodide (1 part per 1,000) was tested for bacteriostatic action against *Staphylococcus aureus, S. typhosus, Proteus proteus, Ps. pyocyaneus,* and *B. subtilis,* with negative results in each case. It has not been possible to have trypanocidal tests carried out here, but preliminary insecticidal tests show that it appears to be an effective stomach poison for house-flies.

The worked described herein has involved much help and co-operation from a number of sources. The author therefore wishes to express grateful acknowledgment to the Keeper of the Herbarium and Library, Royal Botanic Gardens, Kew, for botanical identifications and information; to Drs. E. S. Horgan, R. Kirk and M. H. Sati, and Mr. J. D. Lewis, of the Stack Medical Research Laboratories, for carrying out the biological tests; to the District Commissioner, Yirol, for providing material; to Mr. D. N. Grindley and Riad Eff. Mansour for much assistance on the chemical side; and to the Director, Sudan Medical Service, for permission to publish this communication.

Reference

Ackermann, D., Holtz, F., and Reinwein, H. (1923). *Z. Biol.*, **79**, 113.

Brit. J. Pharmacol. (1948), **3**, 189.

INJECTIONS OF ADRENALINE AND NORADRENALINE, AND FURTHER STUDIES ON LIVER SYMPATHIN

BY

G. B. WEST

From the Pharmacological Laboratory, College of the Pharmaceutical Society, University of London

(Received November 24, 1947)

It is fairly generally conceded that adrenaline injected into the portal vein of mammals causes a smaller rise of blood pressure than when injected into the saphenous, femoral, or jugular veins. This difference between the two modes of administration is less when a large amount of adrenaline is used and it has been shown many times (for references see Dawes, 1946) that it persists when the injection is made very slowly so that it cannot be attributed to retardation of the release of adrenaline into the general circulation. One explanation is that the liver destroys adrenaline, and that the rise of blood pressure after intraportal injection depends on the amount of adrenaline escaping into the general circulation.

The theory that the substance liberated on stimulation of the hepatic nerves in the cat is noradrenaline or some similar substance was put forward by Bacq (1934) and by Stehle and Ellsworth (1937). Greer, Pinkston, Baxter, and Brannon (1937, 1938) have also considered noradrenaline as a possible sympathetic mediator, and have compared the effects of hepatic nerve stimulation (liberating presumably pure sympathin E) with those of injections of adrenaline and noradrenaline in the same animal. In a similar series of experiments, Gaddum and Goodwin (1947) found no evidence against the theory that liver sympathin is noradrenaline. It was of interest, therefore, to observe the effects resulting from intraportal, intrajugular, and intra-arterial injections of *l*-adrenaline and *dl*-noradrenaline in the same animal. In the experiments described below, the activities of the two amines were compared quantitatively with one another by these routes with the object of ascertaining their site of action and fate. It was hoped, also, to obtain further evidence of the similarity of action between injections of noradrenaline and hepatic nerve stimulation.

Dawes (1946) showed that the pressor action of adrenaline injected into the portal vein of a spinal cat was both increased and prolonged by the simultaneous injection of aromatic diamidines and monoamidines, of aliphatic diguanidines, diamidines and monoamidines, and of guanidine itself. This work has been repeated for guanidine, and extended to include the effect of guanidine upon the injection of noradrenaline and hepatic nerve stimulation. Similar experiments with cocaine and ephedrine have also been included.

METHODS

In different experiments, spinal cats, cats anaesthetized with chloralose or urethane, and rabbits under urethane or pentobarbitone were used. Blood pressure records were taken from the carotid artery and injections of the drugs made into the femoral, jugular, or splenic veins, or into the external iliac artery so that the injected solution passed into the vessels of the opposite leg. In some experiments, injections were made into one of the two main splenic arteries. Contractions of the nictitating membrane were recorded isotonically, 7–10 days after denervation by removal of the superior cervical ganglion.

The uterus was fixed at its lower end and its movements recorded directly. Movements of the duodenum were recorded by tying off a segment which was filled with warm saline and connected by a cannula and rubber tubing to a bottle, the upper part of which contained air and was connected to a piston recorder. Solutions of *l*-adrenaline and *dl*-noradrenaline were prepared from the pure bases with $N/100$ HCl.

The hepatic nerves were separated from the hepatic artery and divided centrally. They were stimulated by means of platinum electrodes and an ordinary coil. In most of the experiments with liver sympathin, cocaine hydrochloride (8 mg./kg.) was given intramuscularly.

RESULTS

In confirmation of Dawes (1946), it was found that the relation between doses of adrenaline producing equal rises of blood pressure by the jugular and by the portal routes differed according to the amount injected. The results shown in Table I are

TABLE I

COMPARISON OF EQUI-PRESSOR DOSES OF *l*-ADRENALINE AND *dl*-NORADRENALINE BY THE INTRAPORTAL AND INTRAJUGULAR ROUTES IN CATS

Drug	Intra-portal injection (A) μg.	Intra-jugular injection (B) μg.	Portal (A) / Jugular (B)	Blood pressure rise mm. Hg
l-adrenaline	10	2	5.0	19
	20	5	4.0	40
	40	12.5	3.2	83
	80	27.5	2.9	124
dl-nor-adrenaline	10	5	2.0	41
	20	8	2.5	56
	40	17.5	2.3	95
	80	40	2.0	120

the mean values for 10 cats; it will be seen that when the dose of adrenaline injected into the portal vein is large, proportionately more reaches the general circulation through the hepatic veins and the ratio of portal to equi-pressor jugular dose decreases. On the other hand, the corresponding ratio with noradrenaline remains fairly constant. Why is this difference present? It is well known that both amines, being derivatives of phenyl-ethylamine, are readily destroyed by the liver amine oxidase *in vitro*.

A similar result was found in rabbits under urethane (Table II) although the ratios of portal dose to equi-pressor jugular dose for adrenaline have always been higher than in the cat. For the jugular route in rabbits, adrenaline was found to be a much more active pressor agent than noradrenaline, a fact clearly shown when all the results are plotted graphically (Fig. 1). Great similarity exists between equi-pressor doses of adrenaline and noradrenaline by jugular or splenic vein in the cat

TABLE II

COMPARISON OF EQUI-PRESSOR DOSES OF *l*-ADRENALINE AND *dl*-NORADRENALINE BY THE INTRAPORTAL AND INTRAJUGULAR ROUTES IN RABBITS

Drug	Intra-portal injection (A) μg.	Intra-jugular injection (B) μg.	Portal (A) / Jugular (B)	Blood pressure rise mm. Hg
l-adrenaline	25	2	12.5	24
	50	4	12.5	35
	100	10	10.0	54
	200	25	8.0	80
dl-nor-adrenaline	25	10	2.5	17
	50	20	2.5	24
	100	33	3.0	35
	200	75	2.7	48
	300	100	3.0	60

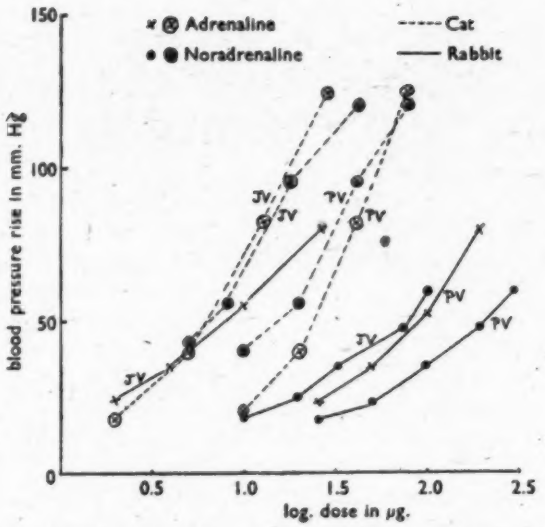


FIG. 1.—The effect of intrajugular (J.V.) and intraportal (P.V.) injections of *l*-adrenaline and *dl*-noradrenaline on the blood pressure rise in cats and rabbits. Note the great similarity between corresponding doses in the cat, and a wide margin between intrajugular doses of the two amines in the rabbit.

(the dotted lines), but a wide margin is indicated between equi-pressor doses by jugular vein in the rabbit. Barger and Dale in 1910 pointed out that adrenaline and other similar methylamino-bases had the property of exaggerating inhibitor as compared with motor effects, whereas the amino- and ethylamino- bases, including noradrenaline, possessed excitor with little or no inhibitory actions. They also noted that a dose of ergotoxine sufficient to reverse the pressor effect of adrenaline in the spinal cat did not reverse that of noradrenaline. The observations reported here may be linked with the fact that in the rabbit, in contrast to the cat, there appears to be no sympathetic depressor component capable of being unmasked by blocking agents such as ergotoxine (Cannon and

TABLE III

RATIO OF DOSE OF *dl*-NORADRENALINE PRODUCING A RISE OF BLOOD PRESSURE OF 48 MM. HG TO EQUI-ACTIVE DOSE OF *l*-ADRENALINE BY THE TWO ROUTES

Animal	No.	Preparation	Mean initial blood pressure mm. Hg	Dose ratio: *dl*-noradrenaline / *l*-adrenaline	
				intra-portal	intra-jugular
Cat	5	Chloralose	118	0.8	0.5
	2	Urethane	65	1.5	1.3
	3	Spinal	62	1.8	1.5
Rabbit	2	Urethane	55	2.0	8.0

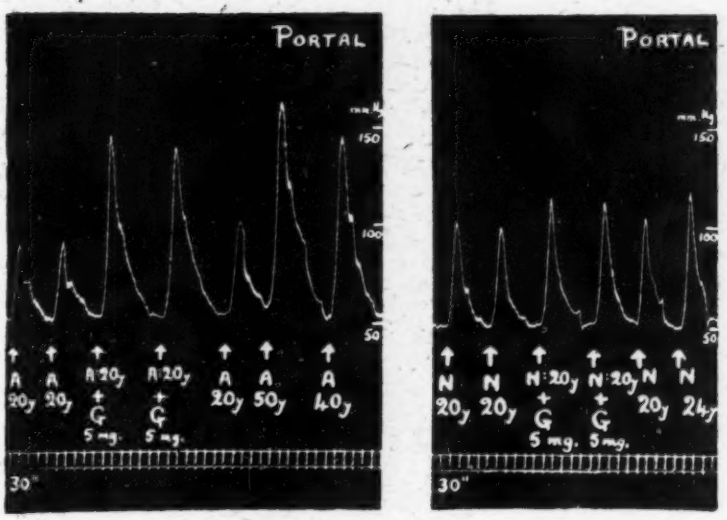


FIG. 2.—Spinal cat. 2.5 kg. Blood pressure record. All injections into the portal circulation; 5 mg. guanidine carbonate (G) greatly increases the pressor action of 20 μg. adrenaline (A) but scarcely affects the pressor action of 20 μg. noradrenaline (N).

Lyman, 1913), and in consequence the ratio of dose of noradrenaline to equi-pressor dose of adrenaline in rabbits is higher than that found in cats. On the other hand, the difference may be related to the initial blood pressure level. For example, as Table III shows, in cats under urethane with low blood pressures, noradrenaline is less active than adrenaline, whereas in cats under chloralose with high blood pressures, it is more active by both routes.

The potentiation of adrenaline injected into the portal circulation

Dawes showed that intraportal injection of amidines and guanidines into spinal cats increased the pressor action not only of intraportal adrenaline but also (and equally well) of the more active sympathomimetic amines, such as corbasil, epinine, and noradrenaline. I have used guanidine only as the potentiating agent, and have compared its effect on adrenaline injections with that on noradrenaline injections. Fig. 2 shows the potentiation of 20 μg. adrenaline, injected into the portal vein of a spinal cat, by 5 mg. guanidine mixed in the same syringe. On intrajugular injection, 5 mg. guanidine itself had no significant effect on blood pressure and showed little or no potentiation of 5 μg. of adrenaline. There is therefore a striking difference between the action of guanidine on adrenaline injected by the portal vein and by the jugular vein. Dawes suggested that the reduction of the inactivation of adrenaline in the liver by guanidine and amidine derivatives was not due to inhibition of mono-amine oxidase, but might be due to some interference with the uptake of adrenaline by the liver cells which prevented it reaching the enzyme.

When these experiments were repeated with noradrenaline, no such potentiation was noted with intraportal doses of guanidine (Fig. 2) or with intrajugular doses of guanidine. The inactivation of intraportal doses of noradrenaline therefore is unaffected by the simultaneous injection of intraportal doses of guanidine. Similar results were recorded in cats under chloralose or urethane and in rabbits under urethane, so that this fact is independent of anaesthetic and is not confined to one species. Dawes showed that if a short interval of time was left between the injection of the guanidine and that of adrenaline the potentiation was less and that it disappeared altogether if the interval was ten minutes. It is possible that the slower-acting noradrenaline may reach the enzyme when the guanidine is partially inactivated. Alternatively, the liver cells may inactivate noradrenaline slowly, especially as it may be that the substance liberated on stimulating the hepatic nerve in the cat is noradrenaline and not adrenaline (Bacq, 1934).

The action of guanidine on intra-arterial injection

It is well known that the injection of adrenaline into the femoral artery causes a much smaller rise of blood pressure than intrajugular or intrafemoral injection. Thus in spinal cats with a cannula in the external iliac artery (so that the injected solution passed into the vessels of the opposite leg), 12 μg. adrenaline were required on intra-arterial injection to cause the same rise of blood pressure as 2 μg. adrenaline by the jugular vein. As with

TABLE IV

COMPARISON OF EQUI-PRESSOR DOSES OF *l*-ADRENALINE AND *dl*-NORADRENALINE BY THE INTRA-ARTERIAL AND INTRAJUGULAR ROUTES IN SPINAL CATS

Cannulae in the external iliac artery and jugular vein

Drug	Intra-arterial injection (A) μg.	Intra-jugular injection (B) μg.	Arterial (A) / Jugular (B)	Blood pressure rise mm. Hg
l-adrenaline	12	2	6.0	28
	25	5	5.0	49
	40	16	2.5	76
dl-nor-adrenaline	10	2	5.0	29
	15	5	3.0	43
	40	16	2.5	62

the splenic vein injections, however, the ratio of equi-pressor doses by the two routes slowly decreased as the blood pressure rise increased (Table IV). When noradrenaline was injected similarly, ratio values slowly decreased, in contrast to fairly constant values after splenic vein injections. It follows that the inactivation processes of adrenaline and noradrenaline are very similar in the limb muscle vessels. Guanidine did not reduce the inactivation of either adrenaline or noradrenaline when injected simultaneously into the femoral artery. In several animals even a depressed action was noted. If vasodilatation caused by guanidine was the principal cause of potentiation in the liver, then one would expect a potentiation on arterial injection. This does not occur and hence small doses of guanidine do not exert an effect on limb muscle vessels.

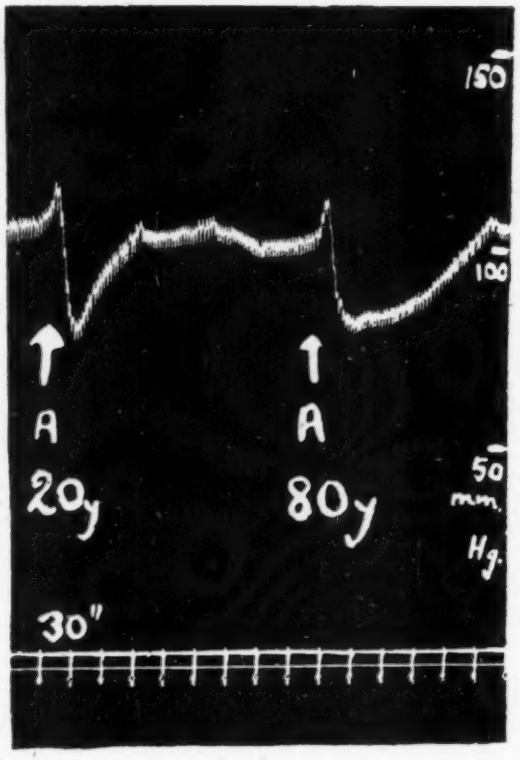


FIG. 4.—Cat. Chloralose. 3.0 kg. Blood pressure record. The effect of adrenaline injected into the splenic artery. Note that the vasodepressor component of 20 μg. is similar to that of 80 μg., but that the latter effect is more prolonged.

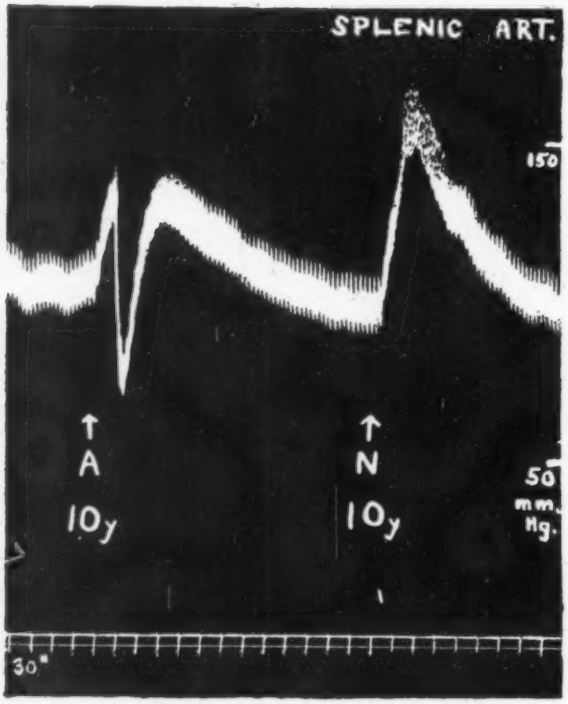


FIG. 3.—The effect on the blood pressure of 10 μg. adrenaline (A) and 10 μg. noradrenaline (N) injected into the splenic artery of a chloralose cat.

When injected into the artery supplying the caudal end of the spleen, adrenaline in small doses caused a small rise followed by a large fall in blood pressure, quite similar to the effect of injecting crude cattle spleen extracts into the jugular vein of cats under chloralose (Euler, 1946a). This depressor component was not completely abolished by intra-arterial doses of atropine, nor was it augmented by eserine. Benadryl in a dose of 0.5 mg. eliminated the depressor action of adrenaline at a time when the normal histamine response (10 μg.) was neutralized. It suggests, therefore, that the injection of adrenaline caused a liberation of histamine in the spleen, but more work must be completed before a definite conclusion can be reached.

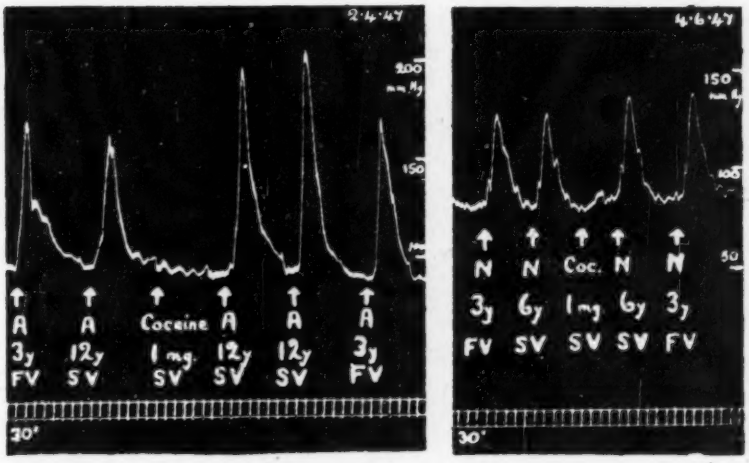


FIG. 5.—Chloralose cats. Blood pressure records. After 1 mg. cocaine hydrochloride injected into the splenic vein (S.V.) 12 μg. adrenaline intraportally is augmented, but not 3 μg. adrenaline and 3 μg. noradrenaline intrafemorally (F.V.) or 6 μg. noradrenaline intraportally.

Noradrenaline, on the other hand, produced a pure rise of pressure at all dose levels (Fig. 3).

This effect of adrenaline on the blood pressure is very striking. It is possible, by using small doses intravenously, to obtain part of the effect (see Gaddum and Goodwin), but the dilatation following injection of 80 μg. into the splenic artery is increased in length but not in depth, when compared with that after 20 μg. adrenaline (Fig. 4), whereas large doses intravenously produce pure vasoconstriction. In addition, these effects are independent of the anaesthetic, since comparable results have been obtained in cats under ether or chloralose. It is worthy of note that the latent period of noradrenaline injections is more than three times that of adrenaline by splenic artery, and this may indicate that the active adrenergic material in the spleen is not noradrenaline, which may have to be methylated or altered before it is effective. A similar longer latent period with noradrenaline than with adrenaline has already been noted (West, 1947a).

Intra-arterial doses of guanidine almost completely removed the depressor component of adrenaline, leaving the vasoconstrictor action, but had no effect on the noradrenaline response. When compared in the same cat under chloralose, equipressor responses were produced by 80 μg. of noradrenaline by splenic artery and 20 μg. by splenic vein (a ratio of 4 : 1), and by 300 μg. of adrenaline by splenic artery and 40 μg. by splenic vein (a ratio of 7.5 : 1). Thus noradrenaline is a much more potent pressor agent by splenic artery than adrenaline, and in addition relatively more adrenaline than noradrenaline is inactivated in the spleen.

The action of cocaine and ephedrine

Experiments similar to those reported for guanidine were completed with 1 mg. doses of cocaine hydrochloride and 100 to 150 μg. doses of ephedrine hydrochloride. Fig. 5 shows the potentiation by intraportal doses of cocaine of intraportal but not intrafemoral adrenaline, and an absence of potentiation of intraportal noradrenaline. This supports the theory that the inactivation of noradrenaline in the liver is a slow process and is not affected by the simultaneous administration of substances such as guanidine and cocaine in small doses. As is well known, cocaine was found to enhance both adrenaline and noradrenaline responses when given by the same routes intrajugularly and intra-arterially (Table V). When used in larger doses, all adrenaline and noradrenaline responses (including intraportal noradrenaline) were enhanced. Ephedrine was an effective enhancing agent by all routes. Thus, there are three types of potentiating agent used in this work: (*a*)

TABLE V

THE EFFECT OF INTRAVENOUS AND INTRA-ARTERIAL GUANIDINE AND COCAINE ON THE PRESSOR ACTIONS OF *l*-ADRENALINE AND *dl*-NORADRENALINE INJECTED BY SIMILAR ROUTES INTO CATS

A = adrenaline. norA = noradrenaline.
+ = potentiation. 0 = no potentiation.
Dose of guanidine was 5 mg. and that of cocaine was 1 mg.

Route of injection	Potentiating agent	Jugular vein		Splenic vein		Iliac artery	
		A	norA	A	norA	A	norA
Jugular vein	Guanidine	0	0	0	0	0	0
	Cocaine	+	+	0	0	0	0
Splenic vein	Guanidine	0	0	+	0	0	0
	Cocaine	0	0	+	0	0	0
Iliac artery	Guanidine	0	0	0	0	Trace	0
	Cocaine	0	0	0	0	+	+

TABLE VI

THE ACTIONS OF *dl*-NORADRENALINE AND *l*-ADRENALINE, INJECTED INTO THE PORTAL AND JUGULAR VEINS, ON THE BLOOD PRESSURE, DENERVATED NICTITATING MEMBRANE, GUT, AND PREGNANT UTERUS OF TWO CHLORALOSE CATS. COCAINE HYDROCHLORIDE (8 MG./KG.) WAS GIVEN INTRAMUSCULARLY

++ = large effect. + = moderate effect. 0 = no effect

Route of injection	Drug	Dose μg.	Mean blood pressure rise mm. Hg	Nictitating membrane rise mm.	Relaxation of gut	Contraction of uterus
Splenic vein ..	Adrenaline	20	70	15	++	++
	Noradrenaline	10	68	13	+	++
Jugular vein ..	Adrenaline	5	70	12	++	++
	Noradrenaline	4	69	5	0	0

ephedrine, acting generally in the body and probably impeding or preventing enzyme inactivation processes when used in certain concentrations; (*b*) cocaine, acting in a manner similar to that of ephedrine but not enhancing intraportal doses of noradrenaline when itself given intraportally in small doses; and (*c*) guanidine, possibly acting directly on the liver cells preventing penetration of adrenaline to the amine oxidase, at the same time producing no potentiation of intraportal noradrenaline.

The effect of adrenaline and noradrenaline on other organs in vivo

Records were taken of the actions of the two amines on the blood pressure, gut, uterus, and denervated nictitating membrane of two pregnant cats. The results of equi-pressor doses by the splenic and jugular veins are shown in Table VI. It is of interest to note that noradrenaline by splenic vein exerted its effects on the membrane, gut, and uterus, i.e., it showed excitor and inhibitor actions, yet an equi-pressor dose by jugular vein gave very feeble actions. It is possible that part of the exogenous noradrenaline may be converted to adrenaline in the liver by N-methylation. Similar results were shown in two spinal non-pregnant cats, intraportal doses of noradrenaline being rather more active on the gut than on the uterus. (Table VII). The fact that noradrenaline in moderate doses may inhibit the non-pregnant uterus and intestine *in vivo* confirms the results of previous workers (Gaddum and Goodwin).

TABLE VII

THE ACTIONS OF *dl*-NORADRENALINE AND *l*-ADRENALINE BY THE INTRAPORTAL AND INTRAJUGULAR ROUTES ON THE BLOOD PRESSURE, GUT, AND NON-PREGNANT UTERUS OF TWO SPINAL CATS

Cocaine hydrochloride (8 mg./kg.) was administered intramuscularly

++ = large relaxation. + = definite but smaller relaxation. 0 = no effect

Route of injection	Drug	Dose μg.	Mean blood pressure rise mm. Hg	Relaxation of gut	Relaxation of uterus
Intraportal	Adrenaline	10	62	++	++
	Noradrenaline	18	60	++	+
Intrajugular	Adrenaline	4	66	+	++
	Noradrenaline	6	66	slight	0

Experiments with liver sympathin

Stimulation of the hepatic nerves in the cat almost invariably caused a rise of blood pressure, part of which was due to constriction of the hepatic artery. The rise occurred even when a clamp on the artery itself raised the blood pressure a few mm. Hg, so that the second effect was due to the liberation of sympathin (Gaddum and Goodwin). Fig. 6 shows the effect of hepatic nerve stimulation before and immediately after the intraportal injection of 5 mg. guanidine carbonate. It will be seen that no potentiation of liver sympathin occurred. In the same animal, adrenaline by the splenic vein was potentiated by guanidine but noradrenaline was not. To overcome any damaging effect of guanidine on the liver cells, the adrenaline responses were completed after liver sympathin and noradrenaline effects had been shown, but the same result was obtained.

In an animal which had not received cocaine, the effect of hepatic nerve stimulation was not potentiated by the simultaneous intraportal injection of cocaine hydrochloride (1 mg.). Gaddum and Goodwin made it clear that cocaine was not essential for work with liver sympathin, since stimulation of the hepatic nerves caused a rise of

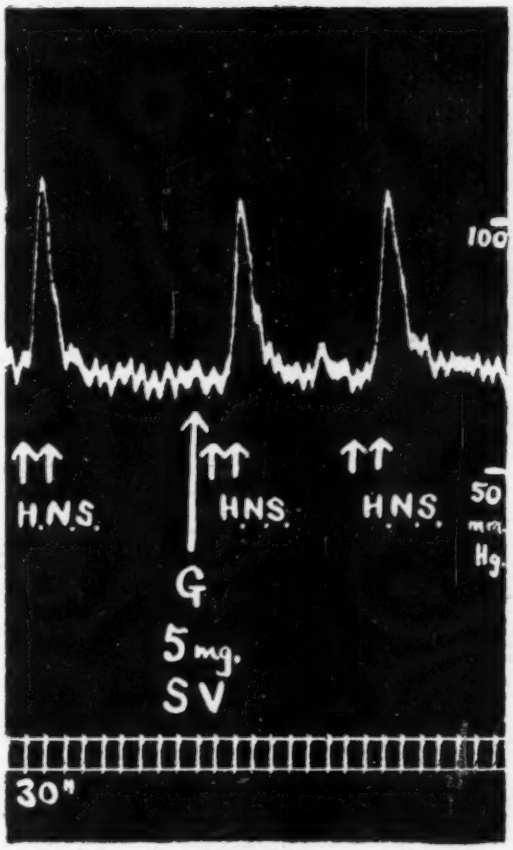


FIG. 6.—Cat 3.6 kg. Chloralose. Cocaine. Blood pressure. Stimulation of hepatic nerves for 30 sec. (H.N.S.). 5 mg. guanidine carbonate injected into the splenic vein (S.V.) 15 sec. before stimulation do not potentiate the pressor action.

blood pressure, contraction of the denervated nictitating membrane, and inhibition of the intestine before cocaine had been given. They stated that whereas cocaine caused a definite increase of the effect on the nictitating membrane, it did not always affect the blood pressure rise. The observations with guanidine and cocaine emphasize the great similarity in action between liver sympathin and noradrenaline (Figs. 2 and 6). Ephedrine potentiated the pressor effect of hepatic nerve stimulation in a feeble way. The actions of liver sympathin on the nictitating membrane and pregnant uterus (excitor responses) and on the gut and non-pregnant uterus (inhibitor responses) agreed very well with those produced by equi-pressor doses of intraportal noradrenaline.

Cannon and Rosenblueth (1937) found that, after the administration of large doses of both ergotoxine (5 mg./kg.) and cocaine to the same cat, the injection of adrenaline caused a pure fall of blood pressure but that stimulation of the hepatic nerves caused a rise. We have observed similar results with ergotamine (3 mg./kg.). By a chance observation, a large dose of dihydroergotamine (2 mg./kg.) was given intraportally in a cat under chloralose. This is about 10 times the normal adrenaline reversal dose, and the effect of subsequent hepatic nerve stimulation surprisingly produced a pure fall of blood pressure (Fig. 7). Likewise, small intraportal doses of noradrenaline produced a fall while corresponding doses of adrenaline had little or no effect. It was not always possible to repeat this result in later experiments. For example, if the dihydroergotamine was given in small increments the normal adrenaline reversal was observed, but after hepatic nerve stimulation or noradrenaline administration a pure rise of blood pressure was produced. Variable results were also produced if the dihydroergotamine was given by the intrajugular route. In addition, intra-arterial noradrenaline gave a rise and intra-arterial adrenaline a fall of blood pressure, whereas intrajugular noradrenaline gave a very small depressor effect. These results only refer to cats under chloralose, and it is hoped to extend this work in order to see if the phenomenon occurs with other anaesthetics. It is of interest to note that dihydroergotamine does not always reverse the adrenaline response in dogs (Orth and Ritchie, 1947).

In two cats, the effects of stimulating the splenic nerve (dissected out in a manner similar to that used for the hepatic nerve) have been recorded. So far, stimulation has produced a pure rise of blood pressure, no depressor component being observed ; the rise although usually small was not potentiated by intra-arterial cocaine or guanidine.

DISCUSSION

For small blood pressure rises in cats, noradrenaline was more active than adrenaline by both the jugular and the splenic routes. As the pressure rise increased, so the sensitivity of the animal increased for adrenaline but decreased for noradrenaline. Ratios of equi-pressor doses of the two amines were thus variable, and this observation may help to explain why such ratios have not been consistent in the past. For example, Crimson and Tainter (1938, 1939) quoted many values for intrafemoral injection ranging from 0.5 to 1.2. For rabbits, noradrenaline was much less active than adrenaline by all routes studied. Two explanations of these phenomena have been recorded: (1) the rabbit appears to have no sympathetic depressor component comparable with that found in the cat, so that the response to adrenaline is pressor only in the rabbit; (2) the

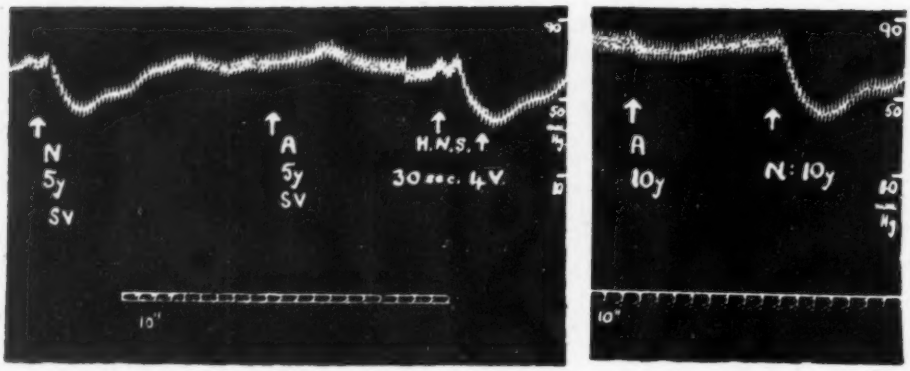


FIG. 7.—Cat. Chloralose. Cocanie. Dihydroergotamine (2 mg./kg.) intraportally. Note that hepatic nerve stimulation (H.N.S. 30 sec.) and intraportal noradrenaline produce depressor effects, whilst intraportal adrenaline scarcely affects the blood pressure.

initial blood pressure levels partially regulate the ratio of equi-pressor doses of the two amines, for in low pressure animals (rabbits under urethane, spinal cats, and cats under urethane) the ratio is higher than 1.0, i.e., the pressor action of noradrenaline is less than that of adrenaline, whereas in high-pressure animals (cats under chloralose) it can be lower. In addition, it has been shown (West, 1947b) that adrenaline and not noradrenaline is a normal constituent of rabbit's blood. It is possible that adrenaline is the normal mediator liberated by adrenergic nerves in the rabbit, but that the mechanism is a little more complex in the cat.

Guanidine on intraportal injection into cats and rabbits potentiated intraportal adrenaline. This potentiation was not shown by guanidine injected into the jugular or femoral veins or into the external iliac or splenic arteries. The effect is thus a local and transitory one on liver cells, presumably preventing the penetration of adrenaline (Dawes, 1946). Noradrenaline in equi-pressor intraportal doses was not potentiated, suggesting that the hepatic inactivation of this amine is not so rapid as with adrenaline. Similarly, suitable intraportal doses of cocaine were found to potentiate intraportal adrenaline but not intraportal noradrenaline, whereas by all other routes studied both were potentiated. Further, intraportal doses of noradrenaline produced effects on the pregnant cat uterus (excitor) and on the gut (inhibitor) when the equi-pressor dose by jugular vein was ineffective. In addition, it has been shown that the inactivation processes for the two amines in the muscle vessels of the limb are similar *in vivo*, since the ratios of equi-pressor doses by the arterial and femoral routes slowly decreased in both cases.

The effects of intraportal noradrenaline have nearly always been similar to those of hepatic nerve stimulation. The simultaneous administration of cocaine and guanidine has not potentiated the pressor effect of stimulation. After large doses of dihydroergotamine by splenic vein, both noradrenaline and hepatic nerve stimulation caused depressor actions when corresponding doses of adrenaline had no effect. In a biochemical study of this and other problems, Blaschko (1942) came to the conclusion on indirect evidence that adrenaline is produced by N-methylation of noradrenaline which may be first formed in the body from tyrosine. It is possible that noradrenaline may be produced in the liver and may act as the adrenergic mediator in that organ, in which case the inactivation process of noradrenaline naturally would be suppressed. A similar position occurs in the spleen, where injections of noradrenaline into the splenic artery and splenic nerve stimulation both produce vasoconstriction, whereas adrenaline injections result in the biphasic response with the marked depressor component. In addition, the ratio of equi-pressor doses of adrenaline by splenic artery and splenic vein is higher (7.5 : 1) than for corresponding doses of noradrenaline (4 : 1). Hence relatively more adrenaline is inactivated in the spleen than is noradrenaline, a result similar to that found for the liver. These observations with the liver and spleen may be linked up with the findings of Euler (1946a, b) that extracts of mammalian spleen, heart, liver, and sympathetic nerves contain a pressor substance with properties like those of noradrenaline or dihydroxy-norephedrine. In addition, he found large quantities of histamine in extracts of mammalian splenic nerves (Euler, 1947). This may account for the depressor component of the adrenaline response after injection into the splenic artery, since it is eliminated by benadryl. More recently, Bacq and Fischer (1947) have reported that extracts of mammalian spleen contain only noradrenaline, extracts of human coronary nerves and arteries only adrenaline, but extracts of mammalian splenic nerves and sympathetic chains a mixture of noradrenaline and adrenaline. Their interpretation of these facts is that in some tissues the synthesis of adrenaline

is stopped at the stage of noradrenaline, whilst in other tissues methylation occurs and adrenaline is formed. They support Euler's suggestion that the term " sympathin " be used for a mixture of varying proportions of adrenaline and noradrenaline.

Summary

1. The pressor effects of *dl*-noradrenaline and *l*-adrenaline, injected into the jugular, femoral, and splenic veins and the splenic and external iliac arteries of cats and rabbits, have been examined.

2. Adrenaline was less active by portal than by jugular vein, though the ratio value for equi-pressor doses by these routes decreased as the pressure rise increased. Noradrenaline was less active by portal than by jugular vein, but the ratio value remained constant.

3. When injected into the portal circulation, noradrenaline was not potentiated by the simultaneous administration of guanidine or cocaine whereas equi-pressor doses of adrenaline were enhanced. Noradrenaline therefore is not rapidly absorbed from the blood stream during its passage through the liver.

4. Intra-arterial and intrajugular injections of adrenaline and noradrenaline were not potentiated by the simultaneous administration of intra-arterial or intrajugular guanidine, but both were enhanced by cocaine.

5. Further similarity in the effects of hepatic nerve stimulation and intraportal injections of noradrenaline have been recorded. Guanidine or cocaine in suitable intraportal doses, for example, do not potentiate the action of liver sympathin. After large intraportal doses of dihydroergotamine, hepatic nerve stimulation and small intraportal doses of noradrenaline produced depressor responses, when corresponding doses of adrenaline were without effect.

6. When injected into the artery supplying the caudal end of the spleen, adrenaline produced a depressor response, possibly due to the liberation of histamine. Noradrenaline, on the other hand, produced a pure rise of blood pressure.

My thanks are due to Mr. K. C. Sparke for technical assistance.

References

Bacq, Z. M. (1934). *Ann. Physiol. Physicochim. Biol.*, **10**, 467.
Bacq, Z. M., and Fischer, P. (1947). *Arch. intern. Physiol.*, **55**, 73.
Barger, G., and Dale, H. H. (1910). *J. Physiol.*, **41**, 19.
Blaschko, H. (1942). *J. Physiol.*, **101**, 337.
Cannon, W. B., and Lyman, H. (1913). *Amer. J. Physiol.*, **104**, 557.
Cannon, W. B., and Rosenblueth, A. (1937). *Autonomic Neuro-effector Systems*. New York: Macmillan Co.
Crimson, C. A., and Tainter, M. L. (1938). *J. Pharmacol.*, **64**, 190.
Crimson, C. A., and Tainter, M. L. (1939). *J. Pharmacol.*, **66**, 146.
Dawes, G. S. (1946). *Brit. J. Pharmacol.*, **1**, 21.
Euler, U. S. v. (1946a). *Acta physiol. scand.*, **11**, 168.
Euler, U. S. v. (1946b). *Acta physiol. scand.*, **12**, 73.
Euler, U. S. v. (1947). *J. Physiol.*, **107**, 10P.
Gaddum, J. H., and Goodwin, L. G. (1947). *J. Physiol.*, **105**, 357.
Greer, C. M., Pinkston, J. O., Baxter, J. H., and Brannon, E. S. (1937). *J. Pharmacol.*, **60**, 108.
Greer, C. M., Pinkston, J. O., Baxter, J. H., and Brannon, E. S. (1938). *J. Pharmacol.*, **62**, 189.
Orth, O. S., and Ritchie, G. (1947). *J. Pharmacol.*, **90**, 166.
Stehle, R. L., and Ellsworth, H. C. (1937). *J. Pharmacol.*, **59**, 114.
West, G. B. (1947a). *J. Physiol.*, **106**, 418.
West, G. B. (1947b). *J. Physiol.*, **106**, 426.

Brit. J. Pharmacol. (1948), **3**, 198.

CIRCULATORY PROPERTIES OF AMIDINE DERIVATIVES I. PRESSOR ANALOGUES OF METHYL *ISO*THIOUREA

BY

F. N. FASTIER

From the Department of Medicine, Otago University, New Zealand

(Received March 6, 1948)

The experiments to be described in this and the succeeding paper are concerned primarily with the circulatory properties of methyl *iso*thiourea in relation to its physico-chemical constitution.

Two considerations prompted such a study: First, the chemical structure of methyl *iso*thiourea is not at all complex; a diversity of related compounds were readily accessible. Secondly, the pharmacological properties by which methyl *iso*thiourea was known to be distinguished (Smirk, 1941; McGeorge, Sherif, and Smirk, 1942) seemed likely to permit the rapid testing of chemically related substances. A pressor action is the most noteworthy of these properties, and, as the substance has been employed therapeutically for maintaining the blood pressure in spinal anaesthesia (Smirk and McGeorge, 1942), the possibility of finding other useful pressor agents was naturally a further inducement to the study of related substances.

Screening experiments involving a wide range of compounds have already received brief comment (Fastier, 1944). Some of the points then raised are dealt with more fully in the present report, whose main purpose it is to outline difficulties in the way of a satisfactory explanation of the distribution of pressor activity among substances chemically related to methyl *iso*thiourea.

METHODS

Commercial samples of iminoazole (Kodak) and 2-aminopyridine (L. Light) were used. S,N-ethylene *iso*thiourea hydrobromide was prepared from β-bromoethylamine hydrobromide and potassium thiocyanate, and S,N-propylene *iso*thiourea hydrochloride by the action of warm concentrated hydrochloric acid on allyl thiourea. The effects of these N-substituted amidine derivatives upon the blood pressure of anaesthetized dogs and cats, the perfusion pressure of pithed rat hind-quarters, and the tonus of isolated strips of rabbit intestine were recorded by methods used in earlier studies (Fastier and Smirk, 1943, 1947).

Methyl *iso*thiourea methylsulphate and methylene di-*iso*thiourea dihydriodide were synthesized by alkylating thiourea with dimethyl sulphate and methylene iodide respectively. Their higher homologues were obtained as hydrobromides by the method of Sprague and Johnson (1937). When no reference to the melting point of the recrystallized salt was found in the literature its composition was checked by a halide determination. Attention was restricted (so far as this paper is concerned) to the blood pressure responses of anaesthetized animals to these other amidine derivatives, except with hexamethylene di-*iso*thiourea; the cardiac effects of the latter were studied (i) in anaesthetized cats by slipping a small glass cardiometer over the heart after the thorax had been opened medially and artificial respiration applied, (ii) in rabbits by perfusing the isolated heart with Ringer-Locke solution by a technique described previously (Fastier and Smirk, 1943), and (iii) in anaesthetized dogs by inserting a long glass cannula into the left jugular vein almost down to the auricle. Clotting was prevented in the last type of experiment by filling the system connecting the cannula to a water manometer with a 5 per cent (w/v) solution of chlorazol fast pink, a little of which was run into the circulation every few minutes. The action of hexamethylene di-*iso*thiourea on the arterial blood pressure of decerebrated and pithed cats, and of anaesthetized and unanaesthetized grey rabbits (method of Grant and Rothschild, 1934), was also investigated, as were its effects on perfused rat hind-quarters and excised rabbit gut.

RESULTS

Influence of chain-length upon pressor activity

One conclusion reached on the basis of the above-mentioned screening experiments is that the pressor activity of basic amidine derivatives is affected adversely by an increase in the length of

side-chains. To discover to what extent "ionic complexity" plays a part in determining activity, twelve homologues of methyl *iso*thiourea of general formula* $CH_3(CH_2)_nS.C(:NH_2^+)NH_2$ have been synthesized and their circulatory effects compared. These alkyl *iso*thioureas provide a series in which a gradual but ultimately considerable variation in the physical properties of the kation is obtained with minimal deviation from the chemical structure of the prototype, methyl *iso*thiourea (for which $n=0$).

The results illustrated in Fig. 1 need little explanation. It will be seen that lengthening the alkyl side-chain produces a notable alteration in the blood pressure response to a moderate initial dose (1–10 mg./kg.) of the *iso*thiourea salt. As n

*Represented conveniently as kations for reasons given before (Fastier and Smirk, 1947).

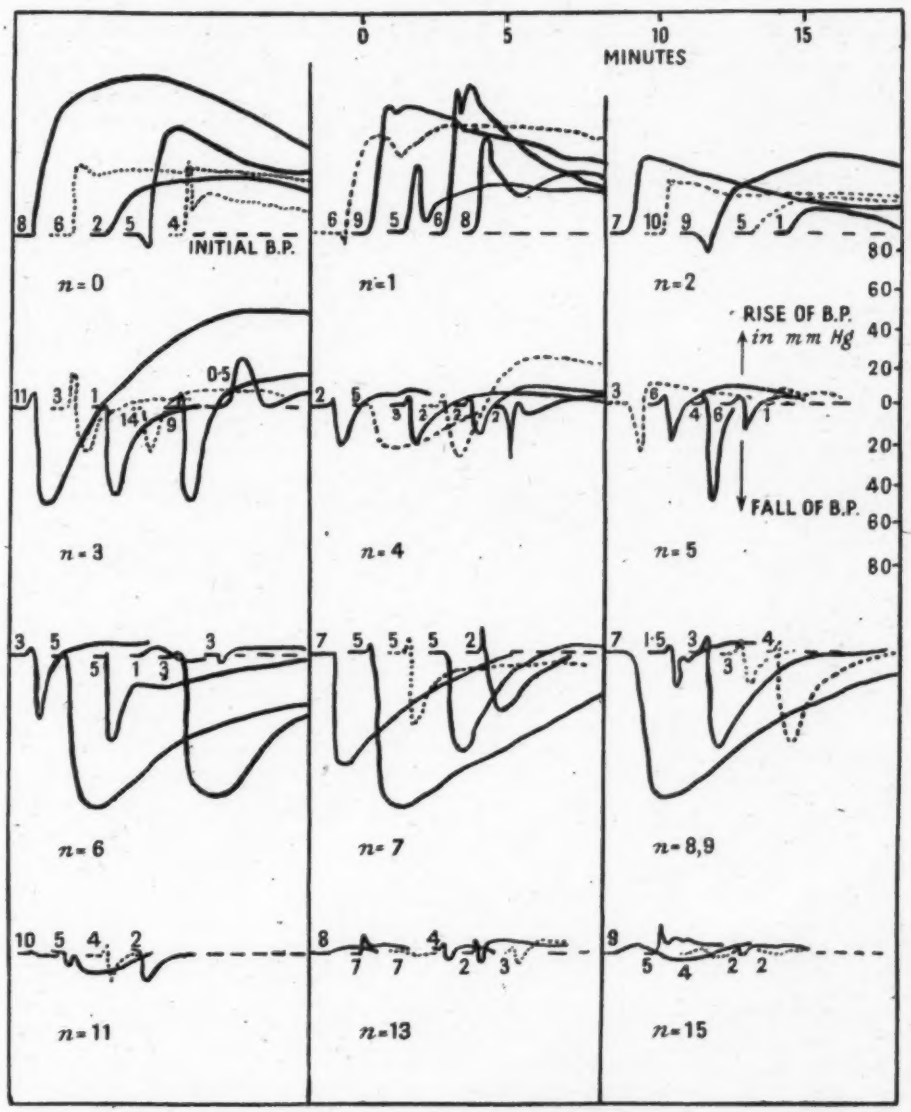


FIG. 1.—Effects on the blood pressure of anaesthetized dogs of *iso*thiourea derivatives of general formula $CH_3(CH_2)_nS.C(:NH_2^+)NH_2$. The curves are facsimiles of kymograph tracings recorded after the intravenous injection of a salt of one of the *iso*thioureas; n refers to the particular one used. The dose in mg./kg. is given alongside each curve. Dotted curves indicate experiments where vagotomy was not performed.

increases, the ability to produce a large, well-maintained rise of blood pressure is soon lost. In contrast to methyl and ethyl *iso*thiourea, the hexyl, heptyl, octyl, and nonyl homologues are predominantly depressor, when given under the same conditions, but the last three tested ($n=11$, 13, 15) are almost inert.

It should be added that the phenomenon of tachyphylaxis, already observed with various other amidine derivatives (Fastier and Smirk, 1943, 1947), was noticed with most of these *iso*thiourea salts. Since the response to successive equal doses of a given *iso*thiourea may change considerably in the course of an experiment and since treatment with one *iso*thiourea often modifies at the same time the response to a related compound no less markedly, it is difficult to make a satisfactory comparison of the effects of several *iso*thiourea salts in

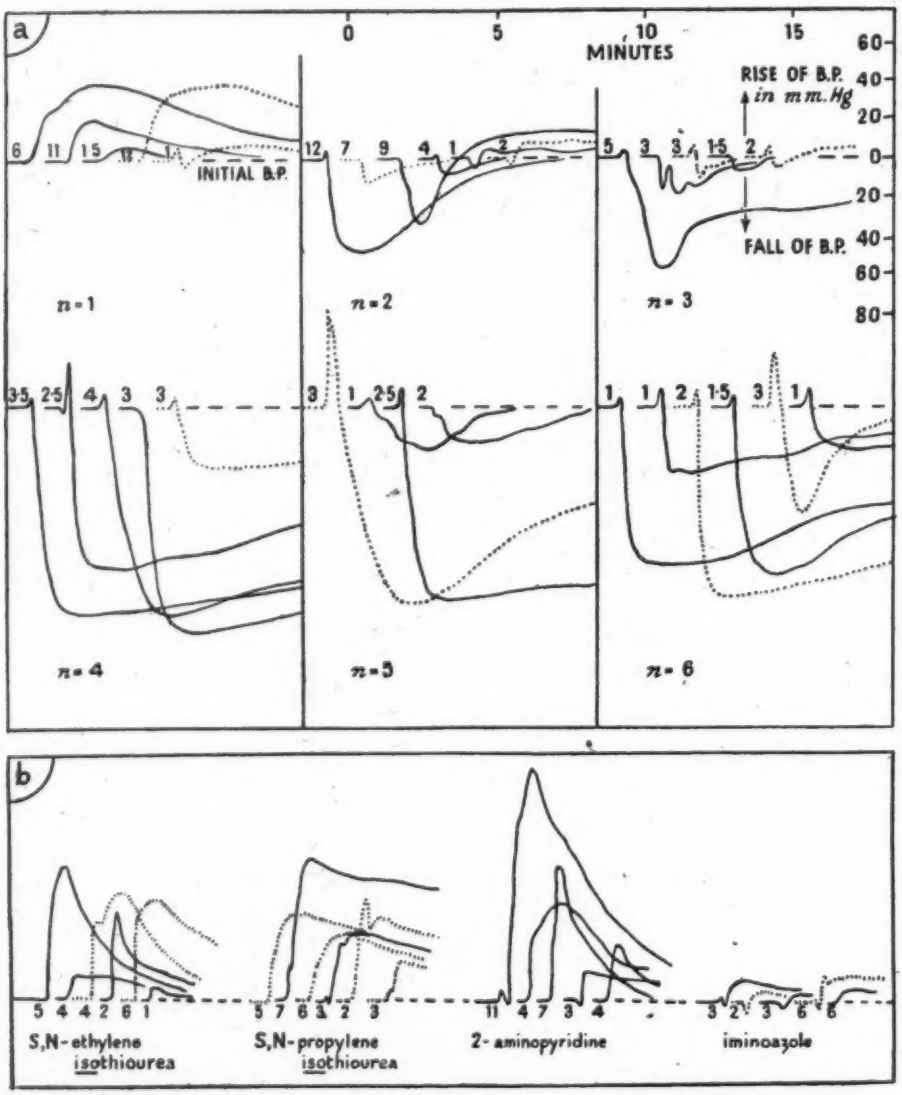


FIG. 2.—Effects on the arterial blood pressure of anaesthetized dogs and cats of (a) di-*iso*thioureas of general formula $H_2N(^+H_2N:)C.S(CH_2)_nS.C(:NH_2^+)NH_2$, and (b) certain N-substituted amidine derivatives. As in Fig. 1, the curves are facsimiles of those recorded after the initial injection of the compound. The dose in mg./kg. is appended to each curve. Experiments on cats are indicated by dotted curves. In (a), *n* refers to the particular di-*iso*thiourea used.

the one animal. For this reason, more reliance has been placed upon a comparison of the effects of initial doses of each *iso*thiourea in fresh preparations.

Depressor action of isothiourea *derivatives, as exemplified by hexamethylene di-*isothiourea *dihydrobromide*

Depressor activity was found to be even more striking in the higher members of the di-*iso*thiourea series of general formula:

$$H_2N(^+H_2N:)C.S(CH_2)_n.S.C(:NH_2^+)NH_2.$$

Only methylene di-*iso*thiourea ($n=1$) possesses pressor activity at all comparable with that of methyl *iso*thiourea (Fig. 2). The interest of the higher members tested ($n=2$–6) lies rather in the sustained falls of blood pressure they can elicit even when injected in fairly small amounts (1–5 mg./kg.). Further experiments were therefore performed

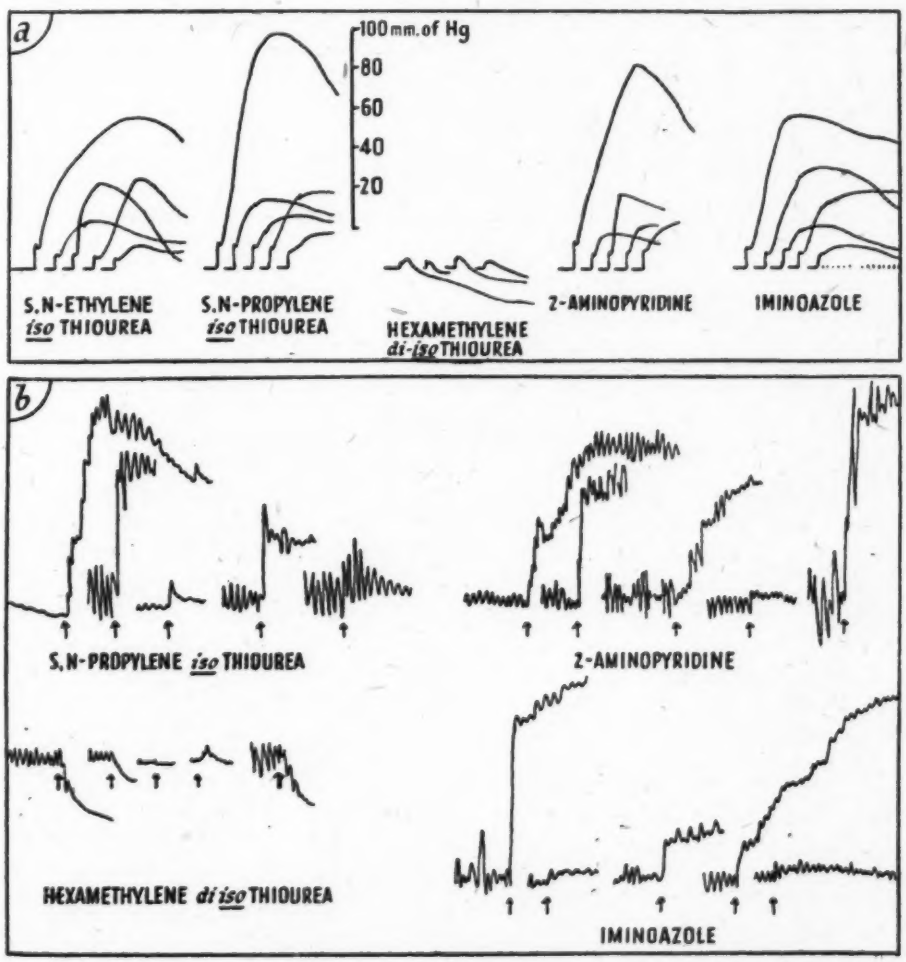


FIG. 3.—Facsimiles of kymograph records showing effects of certain amidine derivatives on (a) pithed rat hind-quarters perfused with Ringer-Locke solution containing sufficient ergotoxine (1: 200,000) to "reverse" the normal vasoconstrictor effect of adrenaline, and (b) excised rabbit intestine rendered insensitive to acetylcholine by atropinization.

The N-substituted amidine derivatives tested constrict the perfused blood vessels and cause the contraction of the gut in the dose given (0.1 c.c. of the *M*/10 solution) even in the presence of these blocking agents. The "higher" amidine derivative hexamethylene di-*iso*thiourea shows little resemblance to them in either preparation when injected in a corresponding dose (0.1 c.c. of the *M*/20 solution).

with the hexamethylene derivative in order to discover how the fall of blood pressure comes about.

In all of 15 animals anaesthetized with sodium barbitone—7 dogs, 6 cats, and 2 rabbits—the intravenous injection of hexamethylene di-*iso*thiourea dihydrobromide caused a persistent fall of blood pressure. The falls obtained in dogs with doses of 0.5–2.0 mg./kg. ranged from 25–110 mm. Hg and lasted for from fifteen to upwards of sixty minutes (Fig. 2). Definite falls of blood pressure were produced in unanaesthetized as well as in anaesthetized grey rabbits.

Effects on cardiac output, so far as they could be discerned, did not suggest that the fall of blood pressure was due to cardiac depression. The *iso*thiourea did not reduce significantly the strength of contraction of isolated rabbit hearts when perfused in concentrations of up to 1 in 25,000; nor was there evidence of depression after its injection in 4 experiments on cats in which cardiometer records were obtained while the blood pressure was falling; rather the reverse. In dogs the venous pressure was lowered by some 10–30 mm. H_2O in 2 out of 4 experiments after a temporary rise. Electrocardiograms taken at the time did not reveal any noteworthy changes in rhythm. The heart rate was usually found to be slowed when noted five to ten minutes after the injection of hexamethylene di-*iso*thiourea, sometimes after an initial acceleration. Vagotomy, which was performed in 7 out of 13 experiments, did not affect the fall of blood pressure.

When a dilator action on blood vessels is thus indicated, it is difficult to prove that the action is a direct one. Hexamethylene di-*iso*thiourea was found to lower the blood pressure in decerebrated cats, but it did not have this effect when the animal was pithed, so long as the blood pressure remained at the low level to which it had fallen subsequent to the latter operation. A vasodilator action was inconspicuous also in the pithed rat hind-quarters preparation except when the pressure had been raised beforehand by treatment with adrenaline or propionamidine. The possibility therefore remains that its vasodilator action in the latter circumstance results from interaction with the vasoconstrictor drug, and not merely from the restoration of some degree of tonus to the blood vessels. It was noted in this connexion that much smaller vasodilator responses to hexamethylene di-*iso*thiourea were obtained when adrenalytic concentrations of ergotoxine were used in place of adrenaline or propionamidine to constrict the perfused rat blood vessels (Fig. 3). A direct inhibitory action on smooth muscle was more definitely suggested by its effect on excised rabbit intestine; in a bath concentration of $M/100{,}000$ or upwards, hexamethylene di-*iso*thiourea was found to depress the tonus and spontaneous movements of the strip (Fig. 3), and to make it at the same time much less sensitive to the action of acetylcholine.

Pressor effects of some N-substituted amidine derivatives

It had been noted on the basis of screening experiments (Fastier, 1944) that pressor activity is distributed fairly widely among amidine derivatives. Inasmuch as the structural relationship to methyl *iso*thiourea (I) of pressor analogues like 2-aminopyridine (III) and iminoazole (IV) is far from close, the possibility must be considered, especially in view of the above results, that their pharmacological resemblance to it is merely superficial. The pressor actions of these particular amidine derivatives have therefore been analysed in some detail, along with those of S,N-ethylene *iso*thiourea (II) and S,N-propylene *iso*thiourea, N-substituted amidine derivatives whose structural relationship to methyl *iso*thiourea is more apparent.

(I) $CH_3.S-C(=NH_2^+)-NH_2$

(II) $CH_2.S-C(=NH^+)-NH_2$, CH_2

(III) $CH=CH-C(=NH^+)-NH_2$, $CH=CH$

(IV) $H-C(=NH^+)-NH-CH$, CH

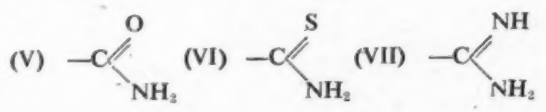
The rises of blood pressure produced in anaesthetized animals by the above bases in doses of the order of 1–10 mg./kg., though less persistent as a rule than those produced by methyl *iso*thiourea under similar conditions, were fairly striking except in the case of iminoazole (Fig. 2b). All four constricted perfused rat blood vessels about as strongly as methyl *iso*thiourea (Fig. 3a), their tonus-increasing effects on this preparation and on isolated rabbit intestine standing in sharp contrast to those of hexamethylene di-*iso*thiourea. Vasoconstrictor effects were not appreciably antagonized by treatment with ergotoxine. Moreover, atropine was usually ineffective in antagonizing their excitatory effects on gut (Fig. 3b).

Discussion

So long as we remain ignorant of the exact site of action of methyl *iso*thiourea we cannot say with certainty that *any* of its chemical relatives act in essentially the same manner, however likely this

may appear when various pharmacological effects are compared. It is tempting to assume that the pharmacological similarity to methyl *iso*thiourea of its various pressor analogues has a chemical basis. Nevertheless such an assumption is reasonable only in so far as it can be shown that these pressor analogues have in common with methyl *iso*thiourea either structural or physical features which are not shared with inactive relatives.

The strong basicity of methyl *iso*thiourea has already been stressed in this connexion (Fastier, 1944). Pressor activity has been found to be inconspicuous, if present at all, in imino-ethers and such other comparatively weakly basic chemical relatives as the ureas, carbamates, thiocarbamates, thioureas, and thiohydantoins which have the amide (V) or thioamide (VI) but not the structurally similar amidine (VII) group. Even amongst the amidine derivatives used in these preliminary experiments, striking pressor activity was observed only with those that ionize freely.



But while possession of a strongly basic character therefore seems to be a necessary condition for pressor activity like that exhibited by methyl *iso*thiourea, it has not proved possible to correlate what appears to be a fairly specific pharmacological action with possession of anything very specific from a structural viewpoint in the way of a " pharmacophoric " or " key " group. In previous reports (Fastier and Smirk, 1943, 1947), it has been shown that pressor activity of apparently the same origin is exhibited not only by homologues like ethyl and *iso*propyl *iso*thiourea but also by various other amidine derivatives of general formula $X.C(:NH_2{+})NH_2$—e.g., methyl *iso*-urea, ethyl *iso*-urea, propionamidine, methylguanidine, and *asym*-dimethylguanidine, where $X=CH_3O$-, C_2H_5O-, C_2H_5-, CH_3NH-, and $(CH_3)_2N$- respectively. Salts of all these bases have been shown to cause, amongst other effects, constriction of perfused blood vessels, even in the presence of strongly adrenalytic concentrations of ergotoxine, and contraction of atropinized gut: evidence which suggests that they owe their pressor activity in part at least to a capacity to constrict blood vessels by a direct action on their musculature. In so far as 2-aminopyridine (III), iminoazole (IV), and other N-substituted amidine derivatives referred to above satisfy the same criteria, their pharmacological resemblance to methyl *iso*thiourea is equally convincing.

It will be noticed that these various musculotropic bases have nothing more in common from a structural viewpoint than the amidine group -C(:NH-+)NH-. How then are we to account for the finding (Fig. 1) that merely lengthening the alkyl side-chain of methyl *iso*thiourea seems to bring about a reversal of activity ?

Presumably, if these pressor analogues of methyl *iso*thiourea do have a fundamentally similar mode of action, we must give chief consideration to " physical " as distinct from purely structural attributes in trying to explain their pharmacological similarity on a chemical basis. The impression that their pressor activity is generally affected adversely by increasing the length of side-chains may therefore be highly significant, and this clue will be followed up in succeeding papers.

Summary

1. In the series of *iso*thioureas of general formula $CH_3(CH_2)_nS.C(:NH)NH_2$, pressor activity is affected adversely by increasing the length of the side-chain (for $n=0$–9). Only the first three members are able to produce large, persistent rises of blood pressure in anaesthetized dogs.

2. Likewise in the di-*iso*thiourea series of general formula $H_2N(HN:)C.S(CH_2)_nS.C(:NH)NH_2$, depressor effects rapidly become predominant as the series is ascended ($n=0$–6). The falls of blood pressure caused by the hexamethylene derivative when given in doses of 2–5 mg./kg. are particularly large and long-lasting; they are brought about mainly, if not entirely, through vasodilatation.

3. Although their structural relationship to methyl *iso*thiourea is much less obvious than that of the above *iso*thioureas, the N-substituted amidine derivatives 2-aminopyridine and iminoazole resemble it closely in their circulatory effects.

4. Like S,N-ethylene and S,N-propylene *iso*thiourea—and also various other amidine derivatives of low molecular weight examined previously (Fastier and Smirk, 1943, 1947)—2-aminopyridine and iminoazole constrict perfused rat blood vessels even in the presence of strongly adrenalytic concentrations of ergotoxine, and cause the contraction of atropinized gut; thus they appear to act directly on the smooth muscle of blood vessels.

5. It is concluded that if the pharmacological resemblance to methyl *iso*thiourea of these various

pressor analogues has indeed a chemical basis, " physical " as distinct from purely structural attributes must play a large part in determining pressor activity in compounds of this type.

This work has been aided by a grant from the New Zealand Medical Research Council. I wish to acknowledge also my indebtedness to Prof. F. H. Smirk for much helpful criticism.

References

Fastier, F. N. (1944). *Nature, Lond.*, **154**, 429.
Fastier, F. N., and Smirk, F. H. (1943). *J. Physiol.*, **101**, 379.
Fastier, F. N., and Smirk, F. H. (1947). *J. Pharmacol.*, **89**, 256.
Grant, R. T., and Rothschild, P. (1934). *J. Physiol.*, **81**, 265.
McGeorge, M., Sherif, M., and Smirk, F. H. (1942). *J. Physiol.*, **100**, 474.
Smirk, F. H. (1941). *Brit. med. J.*, **2**, 510.
Smirk, F. H., and McGeorge, M. (1942). *Lancet*, **2**, 301.
Sprague, J. M., and Johnson, T. B. (1937). *J. Amer. chem. Soc.*, **59**, 1837.

Brit. J. Pharmacol. (1948), **3**, 205.

CIRCULATORY PROPERTIES OF AMIDINE DERIVATIVES
II. POTENTIATION OF THE VASOCONSTRICTOR ACTION OF ADRENALINE

BY

F. N. FASTIER AND C. S. W. REID

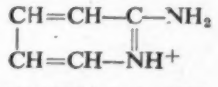
From the Department of Medicine, Otago University, New Zealand

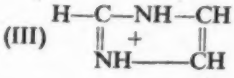
(Received November 11, 1947)

A capacity to enhance the pressor action of adrenaline in anaesthetized animals is almost as conspicuous in methyl *iso*thiourea* (I) and its nearer homologues as their own pressor activity. Both properties have been described before in some detail (McGeorge, Sherif, and Smirk, 1942; Fastier and Smirk, 1943), and evidence has been obtained which points to the blood vessel wall as the main site of action for pressor as well as potentiating effects.

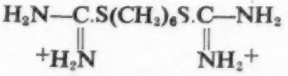
Various other amidine derivatives of fairly low molecular weight have since been found to produce closely corresponding changes in sensitivity to the pressor and vasoconstrictor actions of adrenaline. Results obtained with derivatives of the type $X.C(:NH_2+)NH_2$—e.g., ethyl *iso*-urea, propionamidine, and *asym*-dimethylguanidine, where $X=C_2H_5O$-, C_2H_5-, and $(CH_3)_2N$- respectively—have already been reported (Fastier and Smirk, 1947). That even the presence of substituents in the amidine group itself does not invariably preclude this kind of activity will be shown below for such bases as S,N-ethylene *iso*thiourea, 2-aminopyridine (II), and iminoazole (III).

(I) $CH_3S.C(=NH_2+)-NH_2$

(II) 

(III) 

(IV) $CH_3(CH_2)_8S.C(=NH_2+)-NH_2$

(V) 

Nevertheless "higher" amidine derivatives as a class seem to behave quite differently. So far from enhancing sensitivity to adrenaline when injected in moderately large doses, long-chain amidine derivatives such as *n*-nonyl *iso*thiourea (IV) and hexamethylene di-*iso*thiourea (V) bring about no less emphatic *de*sensitization. Their effects upon the blood pressure, discussed in the preceding paper (Fastier, 1948), appear equally anomalous at first sight. However, a more intensive study of the activity displayed by these compounds has indicated how such contrasting observations may be reconciled; as will also be shown below, an appreciation of the ambivalent character of typical amidine derivatives goes a long way towards explaining differences in effects upon sensitivity to adrenaline, outstanding though these may appear superficially.

* Represented conveniently as a kation for reasons given before (Fastier and Smirk, 1947).

METHODS

Experiments were performed upon dogs and cats anaesthetized with sodium barbitone and upon pithed rat hind-quarters perfused at a constant rate, as described in another paper (Fastier and Smirk, 1947). Ringer-Locke solution aerated with oxygen containing 5 per cent carbon dioxide was used for all perfusion experiments. Salts of the amidine derivatives were dissolved in Ringer-Locke solution to give the dilutions specified in the text, a little alkali being added as required when the resulting solution was acidic.

"Autosensitization"—an increasing sensitivity in the response of the preparation to adrenaline owing to intrinsic causes (*vide* Jang, 1940, and others whom he quotes)—was normally encountered at an early stage of each experiment. However, after some 30–60 min. a stage was usually reached at which the response to adrenaline had become substantially constant, provided that "cumulative" effects were also avoided by keeping the interval between successive injections to 6 or, on occasion, to as much as 9 or 12 min. Even so, the changes in sensitivity brought about by a given amidine derivative were sometimes difficult to assess because, as in the process of autosensitization, the height and the duration of the response to adrenaline were not always equally affected.

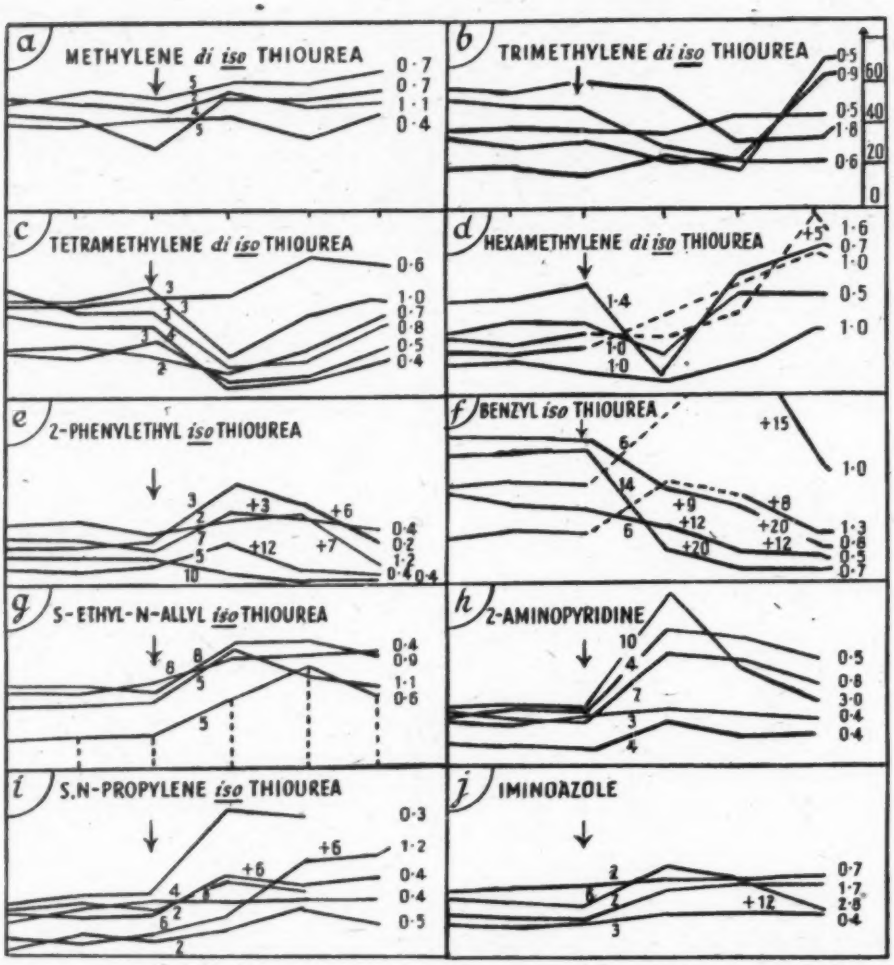


FIG. 1.—Effects of various amidine derivatives upon the sensitivity of anaesthetized dogs to the pressor action of adrenaline. Each "sensitivity" curve was constructed as shown under (g), where the dotted ordinates are the rises of blood pressure caused by the injection of small doses of adrenaline at 6-minute intervals reduced to a common base line. The rise after the heavy arrow, which marks the point of injection of the amidine derivative (dose in mg./kg. given alongside each curve), thus indicates an enhanced response to a fixed dose of adrenaline (dose given in μg./kg. to the right of each curve). Dotted curves (d,f) refer to experiments where the dose of *iso*thiourea was injected very slowly.

The pressure changes due to the amidine derivative itself provided an additional complication. Plotting the heights of the initial pressure response to adrenaline was thought to provide as convenient and reliable an index as any of sensitizing and desensitizing effects, and this has been the procedure adopted in constructing Figs. 1 and 2.

RESULTS

Experiments were first performed on anaesthetized dogs and cats. The results illustrated in Fig. 1 are typical of those obtained. They show that a variety of amidine derivatives are able to enhance the pressor action of adrenaline when their salts are injected in doses of the order of 1–10 mg./kg. They also show, however, that some amidine derivatives normally produce adrenalytic effects when tested under these conditions (Fig. 1c, d, f) and that others are far from constant in effect (Fig. 1b, e).

In order to see whether clear-cut results could be obtained with compounds of the latter type, two or three of them were given in widely graded doses. It so came to be noticed that while desensitization was produced eventually as the dosage was increased, sensitization was usually obtained at an earlier stage of the experiment. Some of the

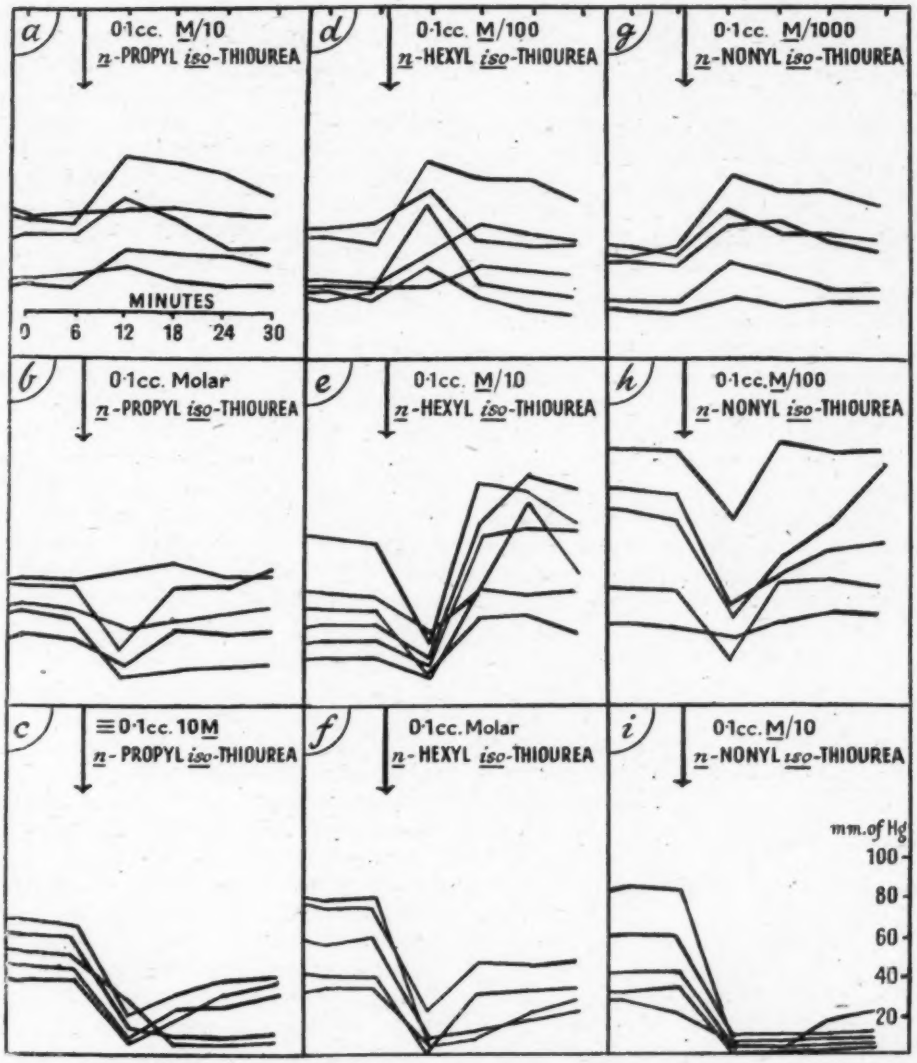


FIG. 2.—Pithed rat hind-quarters preparation. The sensitivity curves shown in this diagram were constructed as indicated under Fig. 1 but illustrate changes in the height of successive *vasoconstrictor* responses to a fixed dose of adrenaline (usually 0.1 μg.) given at 6-minute intervals. Thus in the five experiments represented in the top left-hand corner, the slope of the curves indicates strong sensitization to adrenaline after the injection of a moderate dose of *n*-propyl *iso*thiourea. The same compound can be seen to have produced equally emphatic desensitization in experiments in which larger doses were given (b,c).

Note that both sensitization and desensitization to adrenaline can be produced also by higher homologues in appropriate doses (d,e,f; g,h,i) although the effect of a given dose, say 0.1 c.c. of an *M*/10 solution (a,e,i), varies greatly from one compound to another.

"pure" adrenalytics were then examined. Even these, it was discovered, were quite capable of producing sensitization to the pressor action of adrenaline; a dose which would have caused immediate desensitization if washed into the circulation all at once brought about no less emphatic sensitization, initially at any rate, when administered over a period of 20–30 minutes (Fig. 1d, f). Consequently, the suspicion grew that the response to an amidine derivative might in general be influenced qualitatively as well as quantitatively by dosage. The following experiments were therefore carried out with a more typical compound, effects upon sensitivity to adrenaline being investigated on pithed rat

hind-quarters in preference to anaesthetized animals in order to simplify conditions as far as possible.

Changes in the sensitivity of perfused rat blood vessels to the vasoconstrictor action of adrenaline induced by various doses of n-*hexyl* iso*thiourea hydrobromide*

When small quantities (0.05–0.20 c.c.) of solutions varying in concentration from $M/100{,}000$ to $2M$ were injected one minute before the next dose of adrenaline was due, a threshold for sensitization was found with a concentration of the order of $M/10{,}000$. As the concentration of the *iso*thiourea solution was increased, the sensitization produced increased in extent, becoming maximal for a concentration of the order of $M/100$. The vasoconstrictor response to adrenaline was frequently doubled with this dose (Fig. 2d).

With somewhat larger doses, however, the initial effect was a lesser degree of sensitization; and when the dosage was increased still further, actual desensitization resulted (Fig. 2e, f). The desensitization was usually transient; in such instances (Fig. 2e) a subsequent phase of sensitization followed regularly, which seems highly significant when it is recalled that the perfusion fluid does not circulate in the rat hind-quarters preparation as blood circulates in the intact animal; fresh Ringer solution is constantly being pumped into the dorsal aorta to renew that escaping from severed vessels. If a drug is not held tenaciously by the tissues it must in consequence soon be washed out of the preparation. Presumably, when such a dose as 0.1 c.c. of the $M/10$ solution is injected, the local (i.e., the "effective") concentration of *n*-hexyl *iso*thiourea rises sufficiently high for it to exceed a threshold "desensitizing level." It would appear that this concentration cannot be maintained, however, and as it gradually diminishes, recovery takes place, to be followed by a period of sensitization. Even with strongly adrenalytic doses, which might initially reduce the height of the vasoconstrictor response to adrenaline to one quarter or even one tenth of its former value (Fig. 2f), this pattern was normally followed.

The above interpretation of the form of the sensitivity curves shown in Fig. 2d, e, f, was confirmed by experiments in which *n*-hexyl *iso*thiourea was administered by injecting it into the reservoir of Ringer-Locke solution instead of directly into the perfusion cannula. As but 15–20 c.c. of Ringer-Locke solution entered the preparation each minute, diluting the *iso*thiourea in some 200–400 c.c. of the perfusion fluid ensured that it could not rapidly achieve a high concentration in the tissues even if the amount ultimately reaching the preparation was large. Under these conditions it was found possible to demonstrate that desensitization is definitely preceded by sensitization when a large dose of *iso*thiourea is given (as would be expected if the type of effect produced depends mainly upon "effective" concentration). Moreover, desensitization occurred the more rapidly the higher the concentration of *n*-hexyl *iso*thiourea ($M/20{,}000$, $M/5{,}000$, $M/2{,}500$, $M/1{,}000$) or the faster its rate of perfusion. Recovery followed the reverse pattern. Thus on returning to plain Ringer-Locke solution after perfusing an $M/5{,}000$ solution of *n*-hexyl *iso*thiourea, normal sensitivity was regained within a few minutes and followed by fairly long-lasting sensitization. Only in those experiments in which one of the stronger solutions had been perfused for a considerable time was sensitization not observed after changing back to ordinary Ringer-Locke solution, apparently because the dose had reached a toxic level, judged by the persistent loss of irritability of the preparation.

Sensitizing and desensitizing effects of homologues of n-*hexyl* iso*thiourea*

Nine other *iso*thioureas of general formula $CH_3(CH_2)_nS.C(:NH_2^+)NH_2$ were subsequently tested on the pithed rat hind-quarters preparation, *viz.*, methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl, *n*-heptyl, *n*-octyl, *n*-nonyl, and *n*-decyl *iso*thioureas, for which $n=0$, 1, 2, 3, 4, 6, 7, 8, and 9. Their effects upon sensitivity to adrenaline all seemed to be influenced by dosage in essentially the same manner as those of *n*-hexyl *iso*thiourea (for which $n=5$).

Lower homologues differ from the *n*-hexyl member mainly in that sensitization is obtained over a wider range of concentrations. The doses required for the demonstration of desensitization with *n*-propyl *iso*thiourea (Fig. 2c) or with methyl or ethyl *iso*thiourea are definitely "unpharmacological."

With higher homologues, on the other hand, adrenalytic activity was the predominant feature. As shown in Fig. 2i, the desensitization produced by *n*-nonyl *iso*thiourea in such a dose as 0.1 c.c. of the $M/10$ solution is intense. Nevertheless even *n*-nonyl *iso*thiourea was found capable of producing the opposite effect when given in sufficiently small amount (Fig. 2g), as were also *n*-heptyl, *n*-octyl, and *n*-decyl *iso*thioureas.

Likewise with the anaesthetized dog, in which *n*-nonyl and *n*-decyl *iso*thiourea seemed at first to be purely adrenalytic, it was found possible to demonstrate sensitization by using a very small amount for the first injection and gradually work-

ing up the dose. Still higher members ($n=11$, 13, 15) gave little indication of activity; because of their insolubility they were not tested on the rat hind-quarters preparation. The lower members of the series in doses of the order of 1–10 mg./kg. enhanced the pressor action of adrenaline in anaesthetized dogs, and this property extended well up the series (from $n=0$ to $n=7$).

The pithed rat hind-quarters technique is the better adapted, however, to showing the variation encountered during the ascent of the series. Thus with 0.1 c.c. of the $M/100$ solution as the fixed dose, a gradual increase was apparent in the degree of sensitization produced, a maximum being reached at about the heptyl derivative. At the same time the period of detectable activity increased from some 15–20 minutes to upwards of 30–40 minutes. However, from the propyl derivative upwards, there was an increasing delay before maximum sensitization was observed. The desensitization level was actually exceeded for a time with this dose of *n*-hexyl *iso*thiourea and higher homologues, although in order to prove the point for the heptyl and octyl derivatives the first dose of adrenaline had to be injected as soon as 30 seconds later; desensitization was not observed in those experiments in which the interval was increased to 2 or 3 minutes. Its transitory occurrence was demonstrated even with the hexyl derivative, however, by injecting the adrenaline in a steady stream instead of in doses given at regular intervals, and thus obtaining a continuous measure of sensitivity. No such device was necessary with the highest homologues tested; the desensitization obtained with *n*-nonyl and *n*-decyl *iso*thiourea was considerable and relatively persistent even with this small dose (Fig. 2h).

Sensitizing and desensitizing effects of other amidine derivatives

With the di-*iso*thioureas of general formula $H_2N(+H_2N:)C.S(CH_2)_n S.C.(:NH_2+)NH_2$, increasing the length of the polymethylene chain ($n=1$–6) was likewise found to enhance adrenalytic activity. It has been shown above (Fig. 1c, d) that the tetramethylene and hexamethylene derivatives produce desensitization quite consistently in anaesthetized dogs in doses as low as 2–5 mg./kg. In the rat hind-quarters preparation their adrenalytic activity was equally pronounced. Sensitization of the perfused blood vessels was also obtained with most of these di-*iso*thioureas, although it could not be demonstrated regularly with the tetramethylene and hexamethylene derivatives even when their salts were perfused in high dilution. It was an inconstant phenomenon also with benzyl and 2-phenylethyl *iso*thiourea salts* in the rat hind-quarters preparation. That these particular adrenalytics were able under some conditions at least to cause sensitization as well as desensitization to adrenaline was obvious enough, however, from the results obtained with anaesthetized dogs (Fig. 1e, f) and cats.

A much closer resemblance to lower homologues, like methyl and *n*-propyl *iso*thiourea, was displayed by the N-substituted amidine derivatives S,N-ethylene and S,N-propylene *iso*thiourea, 2-aminopyridine (II), and iminoazole (III). They too produce sensitization in the rat hind-quarters preparation even when injected in fairly large amounts. For the demonstration of desensitization, doses equivalent to 0.1 c.c. of an M-10M solution were required. Much smaller doses sufficed, however, with bases like S-ethyl-N-allyl *iso*thiourea and 2-aminoquinoline which, though allied structurally to the above N-substituted amidine derivatives, have considerably larger kations.

Discussion

Nearly all the amidine derivatives whose activity has been studied in detail have been found capable, like *n*-hexyl *iso*thiourea, of producing either sensitization or desensitization to the vasoconstrictor action of adrenaline, the latter effect becoming predominant sooner or later when the dosage was increased. Differences in their effects appear to be quantitative rather than qualitative; for while the effect of a fixed dose varies greatly from one compound to another, a distinctive pattern can be discerned none the less if the *series of effects* produced by a wide range of doses is made the basis of comparison, as indicated in Fig. 2.

Perhaps the strongest evidence in favour of this idea that both "lower" and "higher" amidine derivatives produce essentially similar sensitizing and desensitizing effects lies in the gradual transition from one characteristic type of behaviour to the other seen during the partial ascent of the two homologous series of *iso*thioureas tested. One plausible objection may be raised, however. Why does the effect produced by a fixed dose alter so considerably during the ascent of a homologous series if all the homologues produce a given effect by the same mechanism?

As Ferguson (1939) has properly emphasized in connexion with toxicity data, we must distinguish between "chemical" and "physical" attributes in comparing the pharmacological activity of two drugs. The fact that one of the "higher" members of an *iso*thiourea series is able to produce the same pharmacological effect (e.g., desensitiza-

tion to the vasoconstrictor action of adrenaline) as one of its lower homologues when it is given in a much smaller dose does not necessarily mean that 1 molecule of the higher homologue is as effective as, say, 10 or 100 molecules of the lower homologue *at the site of action*; it might well be that the distribution co-efficient of the lower homologue was relatively so unfavourable that 10 or possibly 100 times as many molecules of *iso*thiourea had to be present in the enveloping medium (the "external phase") in order to maintain the same number of molecules as the higher homologue at the site of action (the "biophase").

To press the argument a little further, we might interpret the above results as follows: both "lower" and "higher" amidine derivatives are able to influence the sensitivity of blood vessels to adrenaline in a characteristic manner by virtue of a particular *chemical* configuration (the charged amidine "head" of the molecule) which enables them to react with the same receptors; but they differ quantitatively in their effects owing to variation in the *physical* properties of the molecule as a whole, which leads in turn to variation in their capacity to reach the appropriate receptors or remain in contact with them. Evidence believed to favour this possibility will be presented in a later paper.

Summary

1. It has been shown for the first ten *iso*thioureas ($n=0$–9) of general formula:

$$CH_3(CH_2)_nS.C(:NH)NH_2$$

that either sensitization or desensitization to the vasoconstrictor action of adrenaline in the pithed rat hind-quarters preparation may be observed after their administration, according to the experimental conditions employed.

2. Which effect is produced seems to depend mainly upon dosage. Sensitization to a varying extent will occur so long as the local concentration of the *iso*thiourea falls within certain limits (which are characteristic of a given compound); but once the hypothetical upper limit is exceeded, desensitization appears and becomes increasingly emphatic if the local concentration of the base rises still higher.

3. Experiments with various other strongly basic amidine derivatives—e.g., methylene di-*iso*thiourea, hexamethylene di-*iso*thiourea, S,N-propylene *iso*thiourea, benzyl *iso*thiourea, 2-aminopyridine, iminoazole—suggest that they too show a qualitative resemblance to the alkyl *iso*thioureas in producing first sensitization and then desensitization to the vasoconstrictor action of adrenaline when they are given in increasing concentration.

4. Adrenalytic effects are observed more especially with the higher members of a series—e.g., *n*-octyl *iso*thiourea, tetramethylene di-*iso*thiourea, and their near homologues. The desensitization to the pressor action of adrenaline which they produce in anaesthetized dogs probably depends (like the sensitization obtained with smaller doses) in part if not entirely upon some process occurring in the blood vessels themselves, since desensitization to the vasoconstrictor action of adrenaline can be readily demonstrated with these "higher" amidine derivatives in the pithed rat hind-quarters preparation.

5. The influence of chemical structure upon this kind of activity is discussed.

This work was aided by a grant from the New Zealand Medical Research Council. It is a pleasure to acknowledge also our indebtedness to Prof. F. H. Smirk for much helpful criticism and advice.

References

Fastier, F. N. (1944). *Nature, Lond.*, **154**, 429.
Fastier, F. N. (1948). *Brit. J. Pharmacol.*, **3**, 198.
Fastier, F. N., and Smirk, F. H. (1943). *J. Physiol.*, **101**, 379.
Fastier, F. N., and Smirk, F. H. (1947). *J. Pharmacol.*, **89**, 256.
Ferguson, J. (1939). *Proc. R. Soc.*, **127B**, 387.
Jang, C. S. (1940). *J. Pharmacol.*, **70**, 347.
McGeorge, M., Sherif, M., and Smirk, F. H. (1942). *J. Physiol.*, **100**, 474.

Brit. J. Pharmacol. (1948), **3**, 211.

DIAMIDINES AS ANTIBACTERIAL COMPOUNDS

BY

R. WIEN, J. HARRISON, AND W. A. FREEMAN

From the Biological Division, May and Baker Ltd., Essex

(Received November 27, 1947)

Study of the diamidines was first directed to protozoal diseases, their usefulness in bacterial infections being discovered subsequently ; some of our early results on propamidine were cited by Thrower and Valentine (1943), and its value in burns and surface infections has been confirmed. Fuller (1942) showed that Gram-positive were more susceptible than Gram-negative organisms to aliphatic diamidines, and we sought for aromatic diamidines with greater activity against coli, proteus, and pyocyanea as well as against staphylococcus. A preliminary account of our findings has been given in conjunction with clinical trials on two of the new compounds (Wien, Harrison, and Freeman, 1948). Now, while penicillin deals with many infections it has little effect against Gram-negative bacteria, and although streptomycin may be of more value there is still a need for other active compounds: we found certain diamidines which should be of value in local chemotherapy since our experimental studies have had clinical confirmation (Kohn and Cross, 1948).

Compounds.—They conform to the general formula Am.B.X.B.Am in which Am represents an amidine group in the 4 or 4′ position, B a benzene nucleus, and X a direct linkage, either an oxygen atom or an $-O(CH_2)_nO-$ group, where n is an integer from 1 to 10. Since all the derivatives described have substituents in the 2 or 2′ position in the benzene nucleus, they may be referred to for convenience in an abbreviated form, e.g., dibromopropamidine signifying 4:4′ - diamidino - 2:2′ - dibromo-diphenoxypropane, and a list of some of those examined will be seen in Table I. Their preparation and properties (Newbery and Berg, 1945) will be described elsewhere. The isethionates of the compounds are readily soluble, forming colourless, neutral, and stable solutions, but they are precipitated in normal saline ; isotonic solutions may be prepared by the addition of 5 per cent (w/v) dextrose.

Materials and Methods

Antibacterial activity

Organisms.—These were derived mainly from the Lister Institute (for N.C.T.C. numbers see Table II), except for *Staph. aureus,* strain No. 19, which was obtained from a case of osteomyelitis, and strain No. 27, which was obtained from a case of staphylococcal septicaemia.

Media.—Hartley's broth was employed, with the addition of 2 per cent (w/v) glucose for streptococci ; a thioglycollate medium was used for clostridia and human defibrinated blood was used in slide-cell experiments for staphylococci.

Methods.—(1) *Bacteriostatic activity* was measured by means of the two-fold serial dilutional method: a heavy inoculum, one drop of a 1:10 dilution of a 24-hour broth culture, was added to each tube, and the highest dilution completely inhibiting visible growth was recorded after incubation for 18 to 24 hours at 37° C.: these results are marked (*a*) in Table II. Bacteriostatic activity in blood was determined by Fleming's slide-cell method, the staphylococcal inoculum being adjusted to give about 40 colonies in the control cell: the highest effective dilution was read where 5 colonies or less were visible (Freeman, 1948).

(2) *Bactericidal activity* was determined by subinoculations on to trypsin-digest agar: growth on agar after 24 hours' incubation at 37° C. denoted lack of bactericidal activity. These results are marked (*b*) in Table II. The bactericidal properties were investigated also by observing the rate at which *Staph. aureus* and *B. coli* were killed from determinations of the minimal effective bactericidal concentrations at intervals up to 24 hours.

Antifungal activity

Method.—This consisted initially in incorporating serial dilutions of a compound in 2 per cent glucose-agar in small tubes infected with spores, which were then left at room temperature for 5 days. The highest dilution completely inhibiting growth was observed.

More reproducible results were obtained by employing a ditch-plate method.

The petri dishes contained 15 ml. of 2 per cent glucose-agar, and a ditch (3/8 in.) was cut right across the diameter of each agar plate. The ditches were then filled with 2 per cent glucose-agar containing solutions of the compounds to be tested; ten-fold dilutions were tested first and subsequently two-fold dilutions, after the approximate range of activity had been determined. The plates were then dried for one hour at 37° C. Inoculations were made with a platinum needle from ten-day-old glucose-agar slopes. Two species of fungi were employed for each plate, one streak being made of each fungus, crossing the ditch at a right-angle and continuing to the opposite edge of the plate. The plates were incubated at 37° C. for 5 days except for *Hormodendron langeronii*, which grew best at room temperature. *Fungistatic activity* was determined by noting the highest dilution which caused complete inhibition of growth of the fungus on the ditch containing the compound. A control was obtained by observing good growth of the streak on the agar which did not contain any compound. Some zonal inhibition was seen at the edges of the ditch owing to diffusion of the compound into the untreated agar, but these zones were disregarded in the assessment of fungistatic activity.

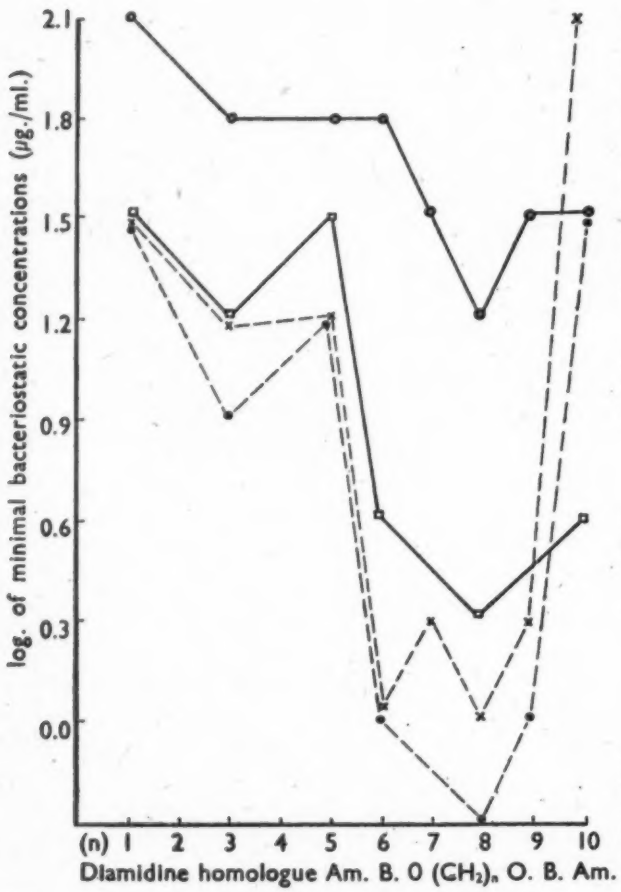


FIG. 1.—Bacteriostatic activities of homologous diphenoxyalkanes expressed as logarithms of minimal bacteriostatic concentrations. Note the increase in effect with increase in the length of the chain to a maximum at $n = 8$. o——o *B. coli* in broth. □——□ *B. flexneri* in broth. x- - -x *Staph. aureus* in blood. ●- - -● *Staph. aureus* in broth.

RESULTS

Bacteriostatic results on homologous diamidines.—Several points are worth noting from the bacteriostatic results shown in Fig. 1. Firstly, there was a graded increase in bacteriostatic activity against staphylococci with increase in the length of the chain to a maximum at $n=6$ to 9, followed by an abrupt decrease at $n=10$; against Gram-negative bacteria the maximum was maintained from $n=6$ to 10. Secondly, activity was retained in the presence of blood. Determination of the toxicities of these compounds on intravenous injection into mice showed an increase in toxicity in ascending the series from the methane (LD50, 30 mg./kg.) to the decane (LD50, 5 mg./kg.) derivative. It should be noted that propamidine, though less active than its higher homologues, was also less toxic to phagocytes (*vide infra*).

Bacteriostatic results on homologous diguanidines.—We found that homologous diguanidines displayed the same gradation in bacteriostatic activity as the diamidines against Gram-positive

TABLE I

BACTERIOSTATIC ACTIVITY OF HALOGENATED DIAMIDINES

Minimal concentrations in μg./ml. for complete inhibition of growth

Compound	*Staph. aureus*		*B. coli*	*Ps. pyocyanea*
	in broth	in blood	in broth	in blood
Phenamidine	128	64	256	512
Iodophenamidine	32	32	64	512
Stilbamidine	32	32	256	256
Iodostilbamidine	8	16	32	64
Bromopropamidine	4	2	32	256
Dibromopropamidine	1	4	4	32
Iodopentamidine	0.5	1.5	64	128
Diiodopentamidine	1	4	8	64
Iodohexamidine	0.5	1	16	8
Diiodohexamidine	1	8	4	16

organisms. Against *Staph. aureus* in blood, in slide-cell experiments, the minimal concentrations inhibiting growth were as follows:—4:4′-diguanidinodiphenoxyethane, 32 μg./ml.; propane homologue, 8 μg./ml.; butane homologue, 4 μg./ml.; pentane homologue, 2 μg./ml.; heptane homologue, 4 μg./ml.; and 256 μg./ml. for the nonane and decane homologues respectively.

Bacteriostatic and fungistatic results on halogenated diamidines.—Table I gives the results obtained with some of the halogenated diamidines examined: the diphenoxyalkanes were bacteriostatically more active than the stilbene compounds, and the diphenyl ethers were much less active. The halogenated diamidines were effective against certain pathogenic fungi, though not more so than the parent compounds; the degree of fungistatic action seemed to depend on the type of fungus, although some of the variation may have been due to the method employed.

Since the diphenoxyalkanes showed promising bacteriostatic activity, various substituted derivatives were examined; it was found that halogen in one or both benzene nuclei had a favourable effect, increasing bacteriostatic activity with little alteration in local toxicity to phagocytes. The mono-halogen derivatives were more active than the di-halogen derivatives against staphylococci, whereas the di-halogen derivatives were more active against Gram-negative bacteria.

Dibromopropamidine and iodohexamidine were found amongst the most effective of the compounds examined, and the results obtained with these and their parent compounds are shown in Table II. For comparison, penicillin (pure sodium salt) was effective in inhibiting the growth of *Staph. aureus* at a minimal concentration of 0.05 μg./ml., and streptomycin was bacteriostatically effective at 8 μg./ml. against *B. coli*, and at 4 μg./ml. against *Ps. pyocyanea*.

Bactericidal results on halogenated diamidines.—The bactericidal activity (results marked (*b*) in

TABLE II

ACTIVITY OF PROPAMIDINE, DIBROMOPROPAMIDINE, HEXAMIDINE, AND IODOHEXAMIDINE AGAINST VARIOUS BACTERIA AND FUNGI

(*a*) = bacteriostatic activity, (*b*) = bactericidal activity, (*f*) = fungistatic activity. Minimal concentrations in μg./ml. for complete inhibition of growth

Organism	N.C.T.C.* strain No.		Propamidine	Dibromo-propamidine	Hexamidine	Iodo-hexamidine
Strep. pyogenes	2432	(*a*)	4	1	0.5	0.5
Strep. viridans	3165	(*a*)	4	2	1	0.5
Staph. aureus	†19	(*a*)	8	1	1	0.5
		(*b*)	16	4	8	4
,, ,, (in 10% serum)	19	(*a*)	16	4	1	1
,, ,, (in blood)	19	(*a*)	16	4	1	1
,, ,, (in blood)	†27	(*a*)	16	16	4	2
Ps. pyocyanea	1999	(*a*)	256	32	16	8
		(*b*)	256	64	32	32
,, ,, (in 10% serum)	1999	(*a*)	256	128	32	32
Proteus vulgaris	3156	(*a*)	128	128	128	128
		(*b*)	256	256	256	256
B. coli	4144	(*a*)	64	4	64	16
		(*b*)	128	32	64	32
,, ,, (in 10% serum)	4144	(*a*)	128	8	64	64
B. flexneri	4835	(*a*)	32	8	4	16
B. enteritidis	4444	(*a*)	256	64	64	16
B. typhi-murium	2110	(*a*)	256	64	64	16
Cl. welchii	273	(*a*)	32	512	256	256
Cl. histolyticum	2915	(*a*)	256	256	256	256
Actinomyces kimberi	4583	(*f*)	100	10	20	20
,, *madurae*	3255	(*f*)	100	50	100	200
,, *hominis*	4525	(*f*)	1000	1000	10	10
Geotrichum dermatitidis	2787	(*f*)	25	200	20	20
Trichophyton tonsurans	2520	(*f*)	100	25	200	200
Hormodendron langeronii	2893	(*f*)	2	500	20	100

* N.C.T.C. (National Collection of Type Cultures). † Laboratory strain number.

Media.—Hartley's broth medium was used for bacteriostatic tests except where indicated; for streptococci 2 per cent (w/v) glucose was added, and for clostridia a thioglycollate medium was employed. Trypsin-digest agar was used for bactericidal tests and 2 per cent glucose agar for fungistatic tests.

TABLE III

ACQUIRED DRUG-RESISTANCE *in vitro*

Bacteriostatic activities: minimal concentrations in μg./ml. for complete inhibition of growth

Compound	Organism	Bacteriostatic activities		No. times increase in resistance	No. sub-cultures
		Initial	Maximal		
		μg./ml.			
Dibromopropamidine ..	*Strep. pyogenes*	1	128	128	24
Propamidine	*Staph. aureus*	8	128	16	10
5-aminoacridine	,, ,,	8	32	4	4
Penicillin	,, ,,	0.03 i.u./ml.	1400 i.u./ml.	46,666	40

Table II) of the halogenated diamidines was somewhat less (four to eight times) than their bacteriostatic activity. From determinations of the rate at which *Staph. aureus* and *B. coli* were killed by various concentrations of the compounds it was found that the maximum bactericidal effect was exerted fairly rapidly, within six to ten hours.

Effect of pH

We confirmed Elson's (1945) observation that the bacteriostatic action of diamidines was decreased in an acid medium and increased in an alkaline medium. At *p*H 6.3, 6.8, and 7.6 the minimal effective concentrations of dibromopropamidine against staphylococci in blood were 64 μg./ml., 4 μg./ml., and 1 μg./ml., respectively, and of propamidine 64 μg./ml., 16 μg./ml., and 4 μg./ml. respectively.

Since the diamidines inhibit the respiration of bacteria (*vide infra*), further evidence was obtained by measuring the oxygen uptake of *B. coli* in phosphate buffer at different *p*H values, in Warburg vessels, with various substrates. After initial incubation (37° C.) with the diamidine for 15 minutes the percentage inhibition of respiration was measured at 10-minute intervals for 2 hours. The manometer cups contained diamidine solution (0.00012 *M*), substrate solution (0.01 *M*), 0.5 ml. washed suspension of *B. coli* containing approximately 10^9 organisms per ml., phosphate buffer (0.033 *M*), and 0.2 ml. of 6 per cent (w/v) potassium hydroxide to absorb carbon dioxide; the volume was adjusted to 3 ml. with saline. The controls did not contain any diamidine. With sodium lactate as substrate, at *p*H 5.6, 6.8, and 7.8, the percentage inhibitions by propamidine after 2 hours were 10, 35, and 72 respectively; for dibromopropamidine they were 0, 51, and 82; for hexamidine 0, 26, and 77, and for iodohexamidine 29, 50, and 87. Similar results were obtained with alanine, glucose, and sodium acetate as substrates. It was evident that acidity decreased and alkalinity increased the inhibitory effect.

Acquired drug-resistance

Resistant-bacteria, whether naturally occurring or developed during treatment with chemical substances, are well recognized and may make selective treatment necessary. Accordingly, serial dilutions of the compounds in broth (2 per cent glucose broth for streptococci) were infected with the organisms (various strains of staphylococci and streptococci isolated from wounds were used), and after incubation at 37° C. for 24 to 48 hours the contents of the tube with the highest concentration of a compound showing growth was used for infecting a further series. The organisms were thus trained by repeated subcultivation to increasing concentrations of the compounds, and the results in Table III show the degrees of resistance attained. The resistance acquired by the various organisms was permanent and fairly readily induced. Cross-resistance experiments were also carried out, and these results are shown in Table IV. The following conclusions can be drawn: (1) staphylococci resistant to penicillin or to 5-aminoacridine were susceptible to diamidines; (2) staphylococci and streptococci resistant to one diamidine were resistant also to other diamidines; (3) staphylococci resistant to diamidines were not resistant to penicillin or 5-aminoacridine. Treatment with diamidines in the human subject might, but would not necessarily, produce similar resistant strains. We encountered some strains of pyocyanea from wounds which, although initially resistant to diamidines, readily lost this resistance when kept for only a week *in vitro* on a drug-free culture medium, showing that the resistance, in this instance, was not permanent. It should be noted that in our experiments the resistance produced by penicillin was much greater than that described by McIntosh and Selbie (1943).

TABLE IV

CROSS-RESISTANCE OF INDUCED DRUG-RESISTANT STRAINS OF *Staph. aureus* AND *Strep. pyogenes*

Figures indicate bacteriostatic activities: minimal concentrations in μg./ml. for complete inhibition of growth

Organism	*Strep. pyogenes*	*Staph. aureus*	*Staph. aureus*	*Staph. aureus*	*Strep. pyogenes*	*Staph. aureus*
Drug-resistance	Dibromopropamidine resistant	Propamidine resistant	Penicillin resistant	5-aminoacridine resistant	Original unmodified strain	Original unmodified strain
Dibromopropamidine ..	128	8	1	1	1	1
Propamidine	256	128	4	16	4	8
Hexamidine	32	8	0.5	1	0.5	1
5-aminoacridine	8	8	8	32	4	8
Penicillin	0.1 i.u./ml.	0.01 i.u./ml.	1400 i.u./ml.	0.01 i.u./ml.	0.03 i.u./ml.	0.03 i.u./ml.

Toxicity and local tolerance (Table V)

(*a*) *Systemic toxicity for mice.*—The LD50 in mice was determined for both the intravenous and subcutaneous routes: symptoms were similar to those previously described for other diamidines (Wien, 1943). Both dibromopropamidine and iodohexamidine had a depressor effect in the chloralose cat; dibromopropamidine increased while iodohexamidine decreased the tone and movements of the isolated rabbit ileum.

(*b*) *Toxicity to human leucocytes.*—Killed staphylococci were added for 30 minutes to citrated human blood previously in contact with diamidine solution for 3 hours at 37° C. After lightly centrifuging the blood in capillary tubes, leucocyte films were prepared from the upper surface of the cellular deposit and stained by Gram's method. The average number of bacteria in 25 phagocytes was counted and the least toxic concentration was noted where the result showed one or less than one coccus per cell.

(*c*) *Toxicity to chick embryo.*—A window was cut in the shell of 10-day-old embryonated eggs, the shell membrane removed, and 0.3 ml. of a solution of a compound (two-fold serial dilutions, using at least 3 embryos for each dilution) dropped on to the collapsed chorio-allantoic membrane. The window was sealed with a waxed coverslip and the mimimum concentration causing death of the embryo within 3 to 4 days was determined.

(*d*) *Effect on guinea-pig skin.*—Intradermal injections (0.05 ml.) were made into the shaved skin of guinea-pigs and the least concentration causing erythema or necrosis was observed. The compounds were tested also for their effect on wound healing (described later), and it was found that there was no delay in healing with concentrations up to 0.4 per cent (in ointment base).

(*e*) *Effect on rabbit conjunctiva.*—Applied under the eyelids of rabbits, solutions of the diamidines caused no irritation of the conjunctiva in concentrations up to 1:1,000.

Antibacterial activity in vivo

By injection.—Although the diamidines display high bacteriostatic and bactericidal activity against

TABLE V

THE SYSTEMIC AND LOCAL TOXICITIES OF PROPAMIDINE, DIBROMOPROPAMIDINE, HEXAMIDINE, IODOHEXAMIDINE, AND 5-AMINOACRIDINE

Compound	Average lethal dose in mg./kg. for mice (*a*)		Minimum toxic concentration (g./100 ml.) for:		
	intravenous	subcutaneous	(*b*) Human phagocytes	(*c*) Chick embryo	(*d*) Guinea-pig skin
Propamidine	42	55	0.6	0.4	0.1
Dibromopropamidine	10	300	0.1	0.8	0.05
Hexamidine	17	62	0.2	0.2	0.025
Iodohexamidine	6	150	0.1	0.4	0.05
5-aminoacridine	15	100	0.01	0.1	0.025

Gram-positive cocci, the ratio of antibacterial activity to systemic toxicity is such that little therapeutic activity can be demonstrated when they are administered parenterally. Hexamidine, given subcutaneously at the maximum tolerated dose, showed only slight therapeutic activity either in prolonging the survival time of mice infected with a virulent strain of staphylococci, or in reducing the incidence of kidney abscesses with a chronic strain. Against salmonella and clostridial infections in mice the results were similarly disappointing. Very slight virucidal activity was shown by hexamidine, but not by the others, against influenza virus infections in mice, but the effect was barely significant.

Locally.—Compounds, in solution and in ointment base, were examined initially for local tolerance on burn-wounds in guinea-pigs by Ungar's (1944) method. It was found that they did not delay the rate of healing after treatment for 4 days at concentrations twice those toxic to phagocytes. Infected wounds were produced on both flanks of the shaved skin of rabbits by scarifying the skin tissue and then infecting with *Staph. aureus* or *Ps. pyocyanea*. The wound was kept moist and enclosed in a plastic cover (Robson, 1946); solutions of the compounds were applied through a hole in the cover which could be closed with a screw. Against staphylococcal infections the diamidines were effective in concentrations of 1 : 1,000, but no effect could be demonstrated at this concentration against pyocyaneal infections. Better results, however, were obtained in the human subject (Kohn and Cross, 1948).

Mode of action

Bernheim (1944) found that propamidine inhibited the oxidative metabolism of bacteria, and we found that the halogenated diamidines had a similar inhibitory effect. We confirmed, also, that a 2-hour period of incubation was necessary in order to obtain maximal effects, probably in order to allow diamidines to penetrate the bacterial cell. In studying the inhibition by diamidines of oxidative systems various substrates were used, in order to discover the effect on the most important dehydrogenases present in the bacterial cell. The substrates used were: glucose, sodium lactate, sodium pyruvate, sodium glutamate, sodium acetate, sodium malate, sodium succinate, and alanine.

The oxygen uptake of *B. coli* was measured in Warburg vessels after a 2-hour period of incubation at *p*H 7.8 in order to obtain optimum effects; the manometer cups contained 0.00012 *M* diamidine solution, 0.01 *M* substrate, 0.5. ml. of a washed bacterial suspension of *B. coli* containing approximately 10^9 organisms per ml., 0.2 ml. of 6 per cent potassium hydroxide, and 0.033 *M* phosphate buffer.

It was found that the diamidines caused marked inhibition and to a similar degree with all the substrates examined. For instance, with glucose and sodium lactate as substrates the percentage inhibitions caused by propamidine after 2 hours at 37° C. were 69 and 72 respectively; for dibromopropamidine the percentage inhibitions were 85 and 87, for hexamidine 83 and 80, and for iodohexamidine 98 and 90. There did not seem to be any relationship between the degree of inhibition and the bacteriostatic activities of these diamidines, although it should be noted that only one organism (*B. coli*) was employed in the manometric experiments.

Similar experiments were carried out with tissue preparations. The substrates were sodium lactate, glucose, sodium succinate, and choline chloride. The lactic and glucose dehydrogenases were obtained from rat brain tissue, and the succinic and choline dehydrogenases were obtained from rat liver. The manometer flasks were set up containing 1 ml. of 0.022 *M* phosphate buffer *p*H 7.8, 0.2 ml. of 0.01 *M* substrate, 0.00012 *M* (and 0.0012 *M*) diamidine solution, Locke's solution, 1 ml. of enzyme preparation, and 0.2 ml. of 6 per cent potassium hydroxide; the volume was adjusted to 3 ml. The flasks were incubated at 37° C. for two hours. No inhibition of oxygen uptake was observed with any of the substrates except choline, with which 72 per cent inhibition was obtained.

In bacteriostatic experiments, with staphylococci in blood, *p*-aminobenzoic acid had no inhibitory effect on the bacteriostatic activity of propamidine, hexamidine, or their halogenated derivatives. But Bichowsky (1944) found that nucleic acid reduced the antibacterial action of propamidine, and Elson (1945) suggested that phospholipides competed with diamidines for the anionic position on the cell. We found in manometric experiments with sodium lactate as substrate that 0.1 per cent of nucleic acid reduced the inhibitory effect of propamidine on the oxygen uptake of *B. coli* by 14 per cent after 1 hour at 37° C. and by 43 per cent after 2 hours. It is suggested, therefore, that the diamidines may act by deranging some phase in the metabolism of bacteria involving nucleic acid.

Discussion

The diamidines are interesting compounds since they have such a wide range of action: they are effective not only against protozoa but also against

bacteria and fungi. The use of the diamidines as local bactericidal compounds requires the fulfilment of certain conditions—i.e., the compounds should show high bacteriostatic and bactericidal activity, they should be effective in body tissues, and they should be well tolerated locally. Tests should parallel as far as possible the conditions of actual use; results obtained, therefore, in serum or blood have more significance than those in broth, and in surface infections local toxicity to tissues is of more importance than systemic toxicity. Although the diamidines are regarded as potentially toxic compounds for parenteral use they are well tolerated when applied locally; the small amounts absorbed from the surface are unlikely to produce any systemic effects.

In the investigation of many substituted diamidines it was found that the introduction of halogen in the 2-position into one or both benzene nuclei had a favourable effect. The introduction of two halogen atoms into the same benzene nucleus in the 2:6 positions was less beneficial—the minimal effective concentration for 2-iodopropamidine against staphylococci in blood was 4 μg./ml., whereas the minimal effective concentration for the 2:6 diiodo derivative was 16 μg./ml. The halogenated stilbene compounds possessed less bacteriostatic activity than the diphenoxyalkane compounds; the halogenated diphenyl ethers were even less active (Table I). We observed that the homologous diguanidines displayed the same gradation in bacteriostatic activity as the diamidines; similar results have been obtained by Fuller (1942) for aliphatic amidines and guanidines.

In an attempt to elucidate their mode of action we found that the diamidines exerted a general inhibitory effect, to a similar degree, on a fairly wide range of oxidizable substrates. The number of substrates involved may be an indication that an effect was exerted directly on a respiratory mediator common to a number of systems, and not on a specific dehydrogenase enzyme system. No parallelism was found between the effects obtained with tissue and bacterial enzymes; there may, however, be some relationship between the effects of the diamidines in causing both an inhibition of choline oxidase and fatty degeneration of the liver. The experiments with induced drug-resistant strains of bacteria threw little light on the problem except to emphasize that dissimilar substances like penicillin and the diamidines probably have different types of action, since an organism made resistant to one diamidine was also resistant to other diamidines but was sensitive to penicillin. Gale (1947) has demonstrated a relationship between the assimilation of glutamic acid by staphylococci and their sensitivity to penicillin, but we have not as yet investigated whether diamidines can similarly block glutamic acid assimilation.

Summary

1. A study of the antibacterial properties of the diamidines showed that bacteriostatic activity in the diphenoxyalkanes rose to a maximum from the propane to the hexane and nonane derivatives. This increased bacteriostatic activity was accompanied by an increase in intravenous toxicity, but by only a relatively small increase in local toxicity to phagocytes. Gram-positive bacteria were more susceptible than Gram-negative bacteria, and the bacteriostatic activity was maintained in the presence of blood.

2. The introduction of halogen into one or both benzene nuclei in the diphenoxyalkanes further increased the bacteriostatic effect against *Staph. aureus* as well as against *B. coli*, *Proteus vulgaris*, and *Ps. pyocyanea*, with little alteration in local toxicity.

3. Two new derivatives, dibromopropamidine and iodohexamidine, were studied more closely, in comparison with their parent compounds, for their possible use in surface infections. They showed both bacteriostatic and bactericidal effects which appear to be due to inhibition of the oxidative metabolism of bacteria. Small differences in *p*H markedly influenced both the inhibitory and the bacteriostatic effects, which were increased in an alkaline medium and decreased in an acid medium.

4. Drug-resistant strains of bacteria could readily be induced by repeated subcultivation *in vitro*; a diamidine-resistant strain, although resistant to other diamidines, was sensitive to penicillin and a penicillin-resistant strain was sensitive to the diamidines.

We are indebted to Miss Pattinson, Mrs. Bradish, and Mrs. Oakley for valuable assistance; to Dr. Gordon and Miss Sowden for the biochemical part of this investigation; to Dr. J. N. Ashley and his colleagues for the preparation of the compounds; to Dr. W. R. Thrower for initiating the clinical trials, and Dr. A. J. Ewins for his interest throughout the investigation.

References

Bernheim, F. (1944). *J. Pharmacol.*, **80**, 199.
Bichowsky, L. (1944). *Proc. Soc. exp. Biol.*, **57**, 163.
Elson, W. O. (1945). *J. infect. Dis.*, **76**, 193.
Freeman, W. A. (1948). *Bull. Inst. med. Lab. Tech.*, **14**, 2.
Fuller, A. T. (1942). *Biochem. J.*, **36**, 548.

Gale, E. F. (1947). *Nature*, **160**, 407.
Kohn, F., and Cross, C. (1948). *Lancet* (in press).
McIntosh, J., and Selbie, F. R. (1943). *Brit. J. exp. Path.*, **24**, 246.
Newbery, G., and Berg, S. S. (1945). British Patent 7258/45.
Robson, J. M. (1946). Personal communication.
Thrower, W. R., and Valentine, F. C. O. (1943). *Lancet*, **1**, 133.
Ungar, J., and Robinson, F. A. (1944). *J. Pharmacol.*, **80**, 217.
Wien, R. (1943). *Ann. trop. Med. Parasit.*, **37**, 1.
Wien, R., Harrison, J., and Freeman, W. A. (1948). *Lancet* (in press).

Since the completion of this paper we have noted an article by Bichowsky-Slomnitzki (*J. Bact.*, 1948, **55**, 33) who found that nucleic acid and certain polyamines (spermine and spermidine) antagonized the inhibitory effect of diamidines (stilbamidine and pentamidine) on the growth of *B. coli*. It was assumed that the diamidines caused metabolic disturbances of the cell nucleotides by fixation of nuclear substances, whereas a different mechanism of action existed in the antagonism between diamidines and polyamines, which was probably associated with their competition for the same cellular substance.

Brit. J. Pharmacol. (1948), 3, 219.

THE ACTION OF THE VENOM OF A MEXICAN SCORPION (*CENTRUROIDES NOXIUS*, HOFFMANN) ON CHOLINESTERASES

BY

E. C. DEL POZO (Mexico City)

From the Physiological Institute, Cambridge

(Received December 4, 1947)

Some of the effects of scorpion venom resemble those of eserine and other cholinesterase inhibiting substances. For instance the venom augments the tension response of skeletal muscle to maximal motor nerve volleys and may produce spontaneous contractions (del Pozo and Anguiano, 1947). This potentiation was shown to be due to repetitive response of the muscle fibres similar to that produced by eserine (Burns and del Pozo, 1947). If an anticholinesterase property of the venom were the cause of this effect, the venom should also produce eserine-like actions on other organs. For instance, substances which act by inhibition of cholinesterase produce in the isolated intestine a characteristic contraction which develops slowly and disappears only gradually when the drug is washed out. The effect contrasts with the immediate and quick contractions produced by drugs acting directly on the smooth muscle. The effect of venom was therefore examined on the isolated intestine of the guinea-pig and rabbit. In addition, *in vitro* experiments were performed to find out if the activity of true- as well as pseudo-cholinesterase could be affected by the venom.

METHODS

Pieces of the guinea-pig's and rabbit's small intestine were suspended in 16 c.c. Mg-free Tyrode solution. The contractions of the fibres of the longitudinal muscle layer were recorded by a Lovatt Evans frontal writing lever. The contractions of the circular muscle layer were observed with the naked eye through the glass wall of the tank, and were seen to cause a lengthening of the preparation. Thus relaxation of the longitudinal muscle layer and contractions of the circular layer both affected the record in the same direction. The bath was emptied by overflow and the substances were added in 0.2–0.5 c.c. saline, with a syringe. Air was bubbled continuously through the Tyrode solution, the temperature of which was kept between 32 and 36° C.

Human serum was used as a source of pseudo-cholinesterase and minced tissue of the caudate nucleus of rabbits as a source of true-cholinesterase. Since human serum contains practically no true- and the tissue of the caudate nucleus practically no pseudo-cholinesterase, their effects on acetylcholine hydrolysis are due in each case to one of the two enzymes only. The effect of venom was compared with that of eserine, which inhibits both cholinesterases (Hawkins and Mendel, 1947).

The procedure adopted was as follows: In preliminary experiments the amounts of cholinesterase preparation necessary to hydrolyse in 10 min. about 90 per cent of 200 or 400 μg. of acetylcholine at 37° C. were determined. Acetylcholine was estimated with the eserinized frog rectus muscle. It was found that 0.2 to 0.25 c.c. human serum or the equivalent of about 10 mg. tissue of the caudate nucleus made up in a final volume of 3 to 3.5 c.c. were required for this purpose. Several samples were then set up simultaneously. Each contained the same amount of the cholinesterase preparation, to which was added either venom or eserine in varying concentrations; the total volume was then made up to 2 or 2.5 c.c. with saline. Ten minutes later the acetylcholine (in 1 c.c.) was added to the samples; these were kept for another 10 min. before the enzyme action was stopped by acidification and boiling. The samples set up with true cholinesterase (a suspension of finely ground brain tissue) were shaken during the whole 20 min. The acetylcholine content of the samples after neutralization was assayed on the eserinized frog rectus muscle. The venom present in some of these samples was found not to interfere with the assay.

The scorpion venom was obtained from ground telsons of a Mexican scorpion (*Centruroides noxius*, Hoffmann) by extraction with saline and precipitation with acetone (Anguiano, 1947). A yellowish greenish powder is obtained which is stable and soluble in saline. The certainly lethal dose of the powder on intravenous injections into 20 g. mice was 1.1 mg.; this dose killed the mice within 15 to 30 min.

RESULTS

1. *Isolated intestine preparations*

Guinea-pig's intestine.—The addition of 2 to 10 mg. of venom extract to the 16 c.c. bath caused,

after a latent period of 30 to 60 sec., a slowly developing contraction of the longitudinal muscle layer. After washing out the venom, relaxation proceeded gradually (Fig. 1b). The effect resembled that of eserine by its long latency, its gradual development and disappearance; it differed, however, from that of eserine in the following respects. After replacing the bath with fresh Tyrode solution the effect of eserine subsided even more gradually that that of the venom extract (see Fig. 1a and b). In addition eserine has a more powerful action on the circular muscle. The preparation, first shortened by eserine owing to the contraction of the longitudinal muscle, frequently shows short periods of lengthening as a result of strong contractions of the circular muscle layer. This is seen in Fig 1a during the first four minutes of the eserine action. With the scorpion venom, contractions of the circular muscle may be absent or occur only once or twice. In the record of Fig. 1b one such period of lengthening owing to contraction of the circular muscle is seen after the administration of the venom.

Approximately 5 mg. of venom were found to have an action equal to or somewhat smaller than that of 10 μg. of eserine; i.e., eserine was about 500 times more active than the venom extract. On repeated administration the venom did not lose its power to cause contraction of the smooth muscle in the intestine; thus no desensitization to the venom took place, unlike the characteristic desensitization which occurs with snake venoms.

Atropine had an antagonistic effect on the action of the venom extract, but the antagonism was less pronounced than with eserine. In the experiment of Fig. 1, atropine 1 in 1.5 million had abolished the action of 5 and even of 10 mg. of venom extract, but 20 mg. of venom extract still produced a pronounced contraction; on the other hand the preparation had become practically insensitive to 150 μg. of eserine (at c). In some experiments one part of atropine in a million reduced but did not abolish the effect of even 5 mg. of venom extract.

Rabbit's intestine.—The effect of the venom extract on the rabbit's intestine resembled that on the guinea-pig's intestine. The addition to the 16 c.c. bath of 1 mg. extract and, in some preparations, even of 0.5 mg. produced small contractions. Stronger effects were obtained with the addition of 5 to 10 mg. of venom extracts. When these amounts were kept in the bath for a few minutes a gradually developing contraction of the longitudinal muscle layer with usually slight contractions only of the circular muscle occurred after a latent period of 30–90 sec. Relaxation of the muscle after washing out the extract proceeded as gradually as after eserine; the venom also produced a contraction of the circular muscle layer shortly after being washed out, but to a less extent than eserine. In nearly all experiments the effect

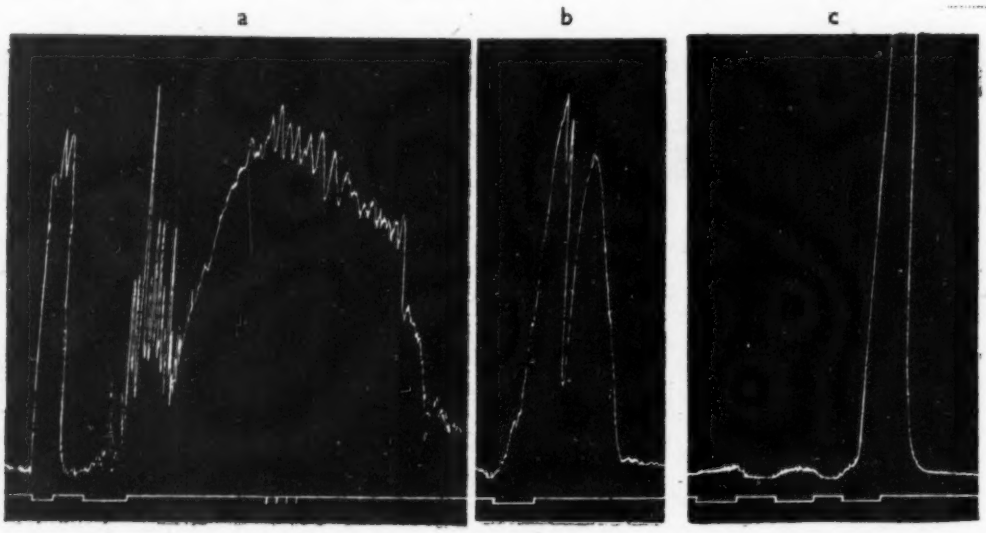


FIG. 1.—Contractions of the isolated guinea-pig's intestine in 16 c.c. bath. *Ac*, acetylcholine washed out after 1 min. *V* and *E*, scorpion venom extract and eserine sulphate respectively washed out after 2 min. *a* and *b* before, *c* during atropine sulphate, 1 in 1.5 million. Time in 30 sec. indicated in *a*. For details see text.)

on the rabbit's intestine of 5 mg. venom extract was slightly weaker, and that of 10 mg. much stronger, than that of 10 μg. of eserine.

Atropine had only a slight antagonistic action on the effect of venom. It was even less effective in antagonizing the action of the venom extract than in the corresponding experiments on the guinea-pig's intestine. The effect of 5 mg. venom extract was only slightly if at all reduced by a concentration of atropine ($1 : 10^6$) which abolished the effect of 10 μg. eserine sulphate.

2. *Cholinesterase preparations*

Venom extract had only a slight inhibiting action on pseudo- as well as on true-cholinesterase. In the experiments of Table I a 0.1 per cent solution of the venom had no effect on the enzyme activity in the samples and 1 to 2 per cent solutions caused partial inhibition. When this effect was compared with the inhibition produced by eserine sulphate it was found that, at room temperature, eserine 1 in 80 millions had a much weaker and eserine 1 in 20 millions a slightly stronger action than 1 to 2 per cent venom extract ; in the one experiment with true-cholinesterase carried out at 37° C. the difference in potency between the two substances was even greater.

Discussion

The extract of venom of the Mexican scorpion used in the present experiments exerted an anti-cholinesterase activity only if tested in very high concentrations, and there was no difference between its effects on pseudo- or true-cholinesterase preparations. When the inhibitory action of the venom extract on these cholinesterases was compared with that of eserine in *in vitro* experiments it was found that, weight for weight, the venom extract was 200,000 to 800,000 times less potent. The necessity of using high concentrations of venom extract for demonstrating its cholinesterase inhibitory action raises the question of how far the effect is due to the venom proper or to impurities present in the extract or, on the other hand, how far such impurities may have reduced any inhibitory action of the venom proper. This question can be answered only if purified preparations of the venom become available.

It is difficult to see how the feeble cholinesterase inhibiting action of the venom extract can contribute more than to a slight extent to the smooth muscle contracting effects on the isolated intestine. The effect has a superficial resemblance to that of eserine, but 0.5 mg. of the venom extract were found to be about as potent as 1 μg. of eserine sulphate, whereas in the *in vitro* experiments on cholinesterase preparations 1 μg. of eserine was as potent as 200 to 800 mg. of venom extract. In addition the smooth muscle contracting effect of the venom was more resistant to atropine than that of eserine. The action of the venom extract on the intestinal preparations appears therefore to be independent of its feeble cholinesterase inhibiting property. Similarly the spontaneous contractions of skeletal muscle, and the potentiating effect which the venom extract has previously been found to exert on the twitch response to single motor nerve volleys in cats, cannot be attributed, or can be attributed to a slight extent only, to inhibition of cholinesterase. These " eserine like " effects of the venom extract can more reasonably be explained as an action either directly on the motor nerve endings or on the motor end plates or on both structures.

TABLE I

COMPARISON OF THE INHIBITORY ACTION OF SCORPION VENOM EXTRACT AND ESERINE SULPHATE ON CHOLINESTERASE ACTIVITY

Cholinesterase source	Temp.(°C.) at which samples were kept	μg. acetylcholine found after 10 minutes action of cholinesterase						
		Concentration of venom extract				Concentration of eserine sulphate		
		0	0.1%	0.33–0.5%	1–2%	1.25×10^{-8}	5×10^{-8}	$1–2 \times 10^{-7}$
(1) Human serum ..	14.5	42	50	75	90	—	150	200
(2) Human serum ..	15.5	21	28	52	110	60	160	200
(3) Caudate nucleus	16.5	9	9	12	45	12	40	150
(4) Caudate nucleus	37.0	80	90	95	110	275	325	325

In experiments (1) to (3) 200 μg. acetylcholine were used as substrate; in (4) 400 μg.

SUMMARY

The effects of extracts of the venom of a Mexican scorpion (*Centruroides noxius*, Hoffmann) were examined on cholinesterase preparations with the view of finding out if the "eserine-like" effects this venom exerts on isolated tissues and in animals on intravenous injections could be explained by a cholinesterase inhibiting property. The venom extract was only found to inhibit pseudo- as well as true-cholinesterase when present in very high concentrations. It is concluded that the symptomatology of the venom poisoning is independent of, or dependent to a very slight degree only on, inhibition of cholinesterase.

I should like to make grateful acknowledgment to Dr. W. Feldberg for his help and interest in this work, to Prof. E. D. Adrian for the hospitality afforded in his Institute, and to the British Council for their invitation to come to England.

REFERENCES

Anguiano, L. G. (1947). *Bol. Inst. Est. Med. Biol.*, **5**, 29.

Burns, L. D., and del Pozo, E. C. (1947). (Unpublished experiments.)

del Pozo, E. C., and Anguiano, L. G. (1947). Abstracts XVII. Int. Physiol. Congress, p. 247.

Hawkins, R. D., and Mendel, B. (1947). *Brit. J. Pharmacol.*, **2**, 173.

Brit. J. Pharmacol. (1948), **3**, 223.

THE ACUTE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED LEAD ACETATE IN NORMAL AND *BAL*-TREATED RABBITS

BY

MICHAEL GINSBURG AND MILES WEATHERALL

From the Pharmacology Department, University of Edinburgh

(Received December 24, 1947)

Little information is available about the actions of 2:3-dimercaptopropanol (BAL) in poisoning by lead salts, and so far as is known nothing has been published about its effect on the distribution of lead in the body. This paper provides some new observations on the distribution of lead, indicated by the use of Pb^{212} (thorium B) as a tracer, and on changes in the distribution after the administration of BAL.

METHODS

Radioactive lead was obtained from the deposit on a charged button exposed to a thorium source. A button with Pb^{212} (intensity of about 1 millicurie) was heated gently in a slightly acidified 0.01 per cent (w/v) solution of lead acetate, so that the Pb^{212} deposit was exchanged for part of the lead in the solution. For administration to rabbits a solution was prepared such that the volume injected (2.0 ml.) contained 2.07 mg. of lead (as acetate) per kg. body weight (i.e. 0.01 mM./kg.) incorporating 100–300 microcuries of Pb^{212} dissolved in 4 per cent (w/v) dextrose. Young rabbits of both sexes and various breeds and of average weight 1.3 kg. were used. Injections were made into the marginal vein of one ear. After injection the rabbits were placed in metabolism cages and allowed access to water but not food. Their bladders were emptied by suprapubic pressure before injection and six, twelve, and eighteen hours afterwards, unless the rabbits were killed earlier. The animals were killed by a blow on the head one, six, or twenty-four hours after the injection of lead acetate. Solutions of BAL were freshly prepared in 66 per cent (v/v) aqueous propylene glycol and were injected into the paravertebral muscles as discussed below.

Immediately after death the thorax was opened, and blood was collected by bleeding from the great veins. Clotting was prevented with heparin, and the samples were centrifuged immediately. The organs were then dissected, starting with those in which a low content of lead was expected, and taking care to avoid contamination between different tissues. Complete organs were washed with water, dried of superficial moisture between filter papers, and weighed; they were then chopped finely and, in organs weighing less than 10 g., ashed entire. From other tissues samples weighing 2 to 10 g. were ashed. Liver samples were taken from well-mixed choppings of the entire organ. Muscle samples were taken from the outer part of the thigh. Bone epiphysis and diaphysis were obtained from the long bones of one hind limb and one fore limb. Marrow was completely removed from the central cavities of these bones and estimated separately. The samples of diaphysis were therefore practically marrow-free; the values for epiphysis, ribs, vertebrae, and skull vault were influenced by the amount of marrow contained in the cancellous tissue. The injected ear and the small piece of cotton-wool used to control bleeding were taken as an independent sample, and in the majority of experiments the uninjected ear was taken as a control. The amount of lead lost by leakage at the site of injection could therefore be assessed; the average amount was slightly more than 2 per cent of the dose. Duplicate samples were taken from the liver and one other organ; from these duplicates an estimate of the error of the lead determinations was made.

After being weighed the tissues were placed in long-necked flasks containing 40 ml. concentrated nitric acid and 20 ml. 60 per cent (w/w) perchloric acid, and allowed to stand for at least 30 minutes. The flasks were then heated cautiously until the contents were boiling, care being taken to prevent the formation of excessive amounts of foam. The tissues dissolved and the solutions were boiled gently until about 20 ml. remained, when a further 30 ml. of concentrated nitric acid was added. Boiling was continued until about 10 ml. of clear solution remained. In all, the solutions were boiled for 1½ to 3 hours. If charring occurred during this process the flasks were allowed to cool and a few drops of fuming

nitric acid or hydrogen peroxide were added until a clear solution was obtained.

The contents of the flasks were washed into beakers and the volume made up to about 100 ml. with water. The reaction of the solution was adjusted to neutrality with 40 per cent (w/v) sodium hydroxide (using B.D.H. Universal Indicator); 0.25 ml. of concentrated hydrochloric acid was then added, followed by 0.5 ml. of 1.12 per cent (w/v) lead acetate. The lead was then precipitated as lead sulphide by the addition of 1.0 ml. of 10 per cent (w/v) sodium sulphide. In order to avoid the precipitation of calcium phosphate from ashed bone samples on the addition of the 40 per cent sodium hydroxide, a slightly different procedure was adopted: the alkali was added until the phosphate began to be precipitated and then just enough concentrated hydrochloric acid to redissolve it was added with vigorous stirring; lead acetate and sodium sulphide were then added as described above.

The precipitates of lead sulphide were allowed to stand for at least one hour before filtering under moderate suction in small Hirsch funnels. The precipitates were collected on small circles of filter paper 2 cm. in diameter. They were washed once with distilled water and once with acetone to carry down the portion adhering to the sides of the funnel and to facilitate drying. The papers were removed when dry and the funnels were washed, first with a small quantity of concentrated hydrochloric acid and then with distilled water. The filtrates and funnel washings were then neutralized, hydrochloric acid, lead acetate, and sodium sulphide were added, and the fresh precipitates were filtered as described above.

The radioactivity of the precipitates so obtained on paper circles 2 cm. in diameter was estimated by means of a Geiger-Müller counter of the usual bell-shaped type with a thin, supported mica window, a paper screen being used to absorb the α particles. The counter was calibrated at each experiment with samples prepared from aliquots of the solution used for injection by precipitation with a lead sulphide carrier as described above. In preliminary experiments it was found that the total count per minute, corrected for the background, was proportional to the amount of radioactive solution used, over the wide range employed in the biological experiments, and that appreciable changes were not produced by doubling or halving the amount of lead carrier used.

From the counts obtained on aliquots of the injected solution and the counts on individual samples under identical conditions, the proportion of the dose per gramme of tissue, and hence the lead concentration per gramme of tissue, were calculated. The total amounts of lead in organs of known weight were calculated from the data, and approximate values were obtained for other tissues on the assumptions that the total blood volume was 70 ml./kg., the total weight of bone marrow 20 g./kg. (Nye, 1931), the total weight of bone without marrow 60 g./kg., the total weight of skin 120 g./kg., and the total weight of skeletal muscle 520 g./kg. (Levine, Mann, Hodge, Ariel, and Du Pont, 1941). With one exception the lead concentration in bone was taken as the value obtained from diaphysis, as being the sample freest from marrow.

By taking two precipitates the error of the estimates was considerably reduced, since a small proportion of the lead sulphide of the first precipitate, lost either because it slipped under the paper or because the particles were too fine to be retained, was recovered in the second precipitate. Also the ratio between the Geiger-Müller counts for the first and second precipitates (generally about 10) gave an indication of the reliability of a particular estimate. The standard error of the lead estimations, calculated from duplicate determinations made during these experiments, was ± 3 per cent. The mean recovery of lead from the entire animal, calculated as described above, was 90 per cent with a standard deviation of ± 10 per cent of the dose.

Results

Data are presented for the distribution of lead in fourteen rabbits, all of which received a single dose of lead acetate containing 2.07 mg. of lead per kg. of body weight and eight of which were treated with BAL. Of the rabbits which did not receive BAL, two were killed at one hour after injection, one at six hours, and three at twenty-four hours. Of the treated rabbits, two were given 50 mg./kg. BAL (i.e. 40 molecules per atom of lead) immediately after the lead acetate and were killed at one hour. One rabbit was given 50 mg./kg. one hour after the lead acetate and 12.5 mg./kg. four hours later and was killed at six hours. Two were treated in the same way but were not killed until twenty-four hours. Three more were treated with BAL 50 mg./kg. nineteen hours after the lead acetate and 12.5 mg./kg. at twenty-three hours and were killed at twenty-four hours. The doses of BAL used were very near the toxic range, as maximal effects were being sought: one rabbit of the last group (No. 93) died nearly twenty-three hours after receiving lead acetate, shortly before the small injection of BAL was due. Ill effects were not observed in any of the other rabbits.

The concentrations of lead in microgrammes per gramme of tissue and the percentages of the dose found in various organs and calculated for various tissues are shown in Tables I and II. Considerable variation occurred between different rabbits which received the same treatment, especially at twenty-four hours, but from the general trend of results it appeared that with a few exceptions the distribution of lead when BAL was not given did not alter greatly between one and twenty-four hours. The exceptions were the plasma and lungs in which the lead content showed a steady decline, and in the contents of the alimentary canal, in which the amount of lead increased in the longer experiments. Changes in other organs did not exceed the variation

TABLE I

THE EFFECT OF TREATMENT WITH BAL ON THE CONCENTRATION OF LEAD IN THE TISSUES OF RABBITS ONE HOUR, SIX HOURS, AND TWENTY-FOUR HOURS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2.07 MG. Pb/KG.)

Time after giving lead:	μg. lead per g. fresh weight of tissue													
	1 hour				6 hours		24 hours							
Treatment with BAL (intramuscular injection):	None		50 mg./kg. at once after lead		None	50 mg./kg. at 1 hr. 12.5 mg./kg. at 5 hr.	None			50 mg./kg. 1 hr. 12.5 mg./kg. 5 hr. after lead		50 mg./kg. at 19 hr. and 12.5 mg./kg. at 23 hr. after lead		
Rabbit No.:	74 ♂	76 ♀*	75 ♀	79 ♂	83 ♀	82 ♀	86 ♀	90 ♂	91 ♂	92 ♂	95 ♀	93† ♂	94‡ ♀	97 ♀
Weight, kg.:	1.20	1.70	1.15	1.22	1.10	1.05	1.40	1.00	0.98	1.15	1.50	1.25	1.25	1.30
Plasma ..	0.50	0.82	2.67	3.17	<0.005	0.18	<0.02	<0.04	0.05	<0.01	<0.31	0.40	<0.15	0.11
Erythrocytes ..	1.49	1.71	0.88	1.78	1.99	0.46	1.65	1.38	1.94	0.29	1.61		<0.30	0.40
Spleen	43.0	30.0	4.74	0.98	32.5	37.6	29.3	2.98	—	10.5	15.5	34.1	27.2	22.8
Bone marrow ..	16.6	17.1	8.39	7.90	26.4	13.7	25.4	9.65	7.18	7.86	31.7	13.4	6.47	12.1
Liver	46.6	38.8	20.4	18.3	22.6	27.3	23.0	52.8	46.2	35.7	12.2	18.5	33.2	24.4
Bile	0.20	0.18	8.55	0.20	3.00	8.17	9.10	0.80	7.30	0.83	10.9	29.5	1.14	7.67
Stomach ..	0.15	0.18	0.93	0.53	0.20	0.35	0.26	0.09	—	1.14	0.87	0.49	1.71	1.26
,, contents	—	—	—	—	0.02	0.04	0.87	<0.01	—	1.29	1.84	0.49	<0.06	0.30
Small intestine	0.41	0.28	3.81	10.8	0.05	1.56	0.46	0.40	0.45	0.24	0.53	4.24	1.42	1.51
,, ,, contents					0.66	2.17	2.12	0.56	—	3.12	2.86	7.25	4.35	4.32
Colon	0.22	—	0.41	0.56	0.19	0.37	0.20	0.29	—	0.29	1.55	0.39	0.36	0.59
,, contents	0.02	0.03	0.39	0.37	0.37	10.5	0.29	1.49	—	3.93		2.35	1.73	—
Kidneys ..	5.31	1.84	12.0	10.0	2.43	3.00	2.22	1.63	4.10	1.52	2.95	2.95	3.15	2.23
Lungs	5.30	3.75	0.99	1.10	3.67	3.11	0.75	1.66	1.16	3.99	4.97	3.60	42.7‡	1.85
Heart	0.16	0.23	0.60	0.79	0.17	0.39	0.17	0.31	0.26	0.48	—	0.75	0.49	—
Skeletal muscle	0.05	0.03	0.25	0.41	0.03	0.24	0.03	0.08	0.01	0.15	<0.09	0.21	0.32	0.09
Diaphyses ..	2.57	1.66	0.64	0.80	3.71	2.76	0.41	1.48	—	1.79	4.66	5.05	2.25	3.45
Epiphyses ..	3.77	3.91	2.40	2.61	8.22	4.24	—	—	8.72	—	5.43	—	5.05	—
Ribs	4.15	4.44	2.98	0.86	—	—	—	1.14	—	—	4.97	—	5.44	2.58
Vertebrae ..	4.72	—	—	—	—	—	—	—	5.96	—	6.83	—	—	—
Skull vault ..	3.60	3.51	1.04	1.86	—	—	—	—	10.4	—	—	—	—	—
Brain	0.07	0.07	0.07	0.16	0.17	0.07	0.07	—	0.08	—	0.29	0.07	—	0.08
Skin	0.10	0.23	0.20	0.23	0.29	0.17	0.10	0.25	0.32	<0.01	—	0.44	0.31	—

* Pregnant. † Died 22 hr. 40 min. after injection of lead acetate. ‡ The injected solution contained some particulate matter, presumably basic lead acetate.

TABLE II

THE EFFECT OF TREATMENT WITH BAL ON THE DISTRIBUTION OF LEAD IN RABBITS ONE HOUR, SIX HOURS, AND TWENTY-FOUR HOURS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2.07 MG. Pb/KG.)

Time after giving lead:	Percentage of dose in entire organ or tissue													
	1 hour				6 hours		24 hours							
Treatment with BAL (intramuscular injection):	None		50 mg./kg. at once after lead		None	50 mg./kg. at 1 hr. 12.5 mg./kg. at 5 hr.	None			50 mg./kg. 1 hr. 12.5 mg./kg. 5 hr. after lead		50 mg./kg. 19 hr. 12.5 mg./kg. 23 hr. after lead		
Rabbit No.:	74 ♂	76 ♀*	75 ♀	79 ♂	83 ♀	82 ♀	86 ♀	90 ♂	91 ♂	92 ♂	95 ♀	93§ ♂	94‖ ♀	97 ♀
Weight, kg.:	1.20	1.70	1.15	1.22	1.10	1.05	1.40	1.00	0.98	1.15	1.50	1.25	1.25	1.30
Plasma	1.25	1.45	4.48	6.22	<0.01	0.43	<0.03	<0.09	0.10	<0.005	<0.66	1.51	<0.36	0.21
Erythrocytes	1.69	2.77	1.48	2.54	3.32	0.54	1.83	1.67	2.42	0.37	2.03		<0.36	0.59
Spleen	2.60	0.43	0.26	0.05	1.14	0.87	0.81	0.16	—	0.31	0.25	0.66	0.42	0.28
Bone marrow	16.1	16.8	8.12	7.63	25.5	13.2	24.6	9.32	7.08	7.64	30.6	13.0	6.25	11.6
Liver	67.7	56.8	40.1	33.0	50.3	60.8	38.1	71.1	51.9	43.5	20.3	31.9	48.8	45.2
Bile	0.006	0.007	0.11	0.006	0.029	0.23	0.22	0.035	0.29	0.017	0.49	1.71	0.058	0.095
Stomach	0.09	0.10	0.85	0.36	0.14	0.30	0.17	0.06	—	0.72	0.51	0.31	0.62	1.00
,, contents	—	—	—	—	0.04	0.10	1.28	<0.01	—	1.29	4.10	1.26	<0.16	0.73
Small intestine	0.32	0.37	6.40	20.3	0.07	2.06	0.56	0.52	0.66	0.27	0.59	3.72	1.84	2.83
,, ,, contents					0.29	0.95	0.81	0.11	—	0.67	0.62	3.12	1.11	1.58
Colon	0.11	—	0.26	0.30	0.10	0.16	0.07	0.10	—	0.07	0.50	0.11	0.14	0.24
,, contents	0.013	0.006	0.10	0.029	0.17	0.77	0.50	0.21	—	0.61		0.28	0.51	—
Kidneys	1.50	0.52	4.02	4.00	0.65	0.92	0.75	0.49	1.27	0.38	0.74	0.82	1.00	0.60
Lungs	1.13	0.62	0.25	0.44	0.42	0.63	0.38	0.52	0.44	0.41	0.31	0.63	11.7‖	0.32
Heart	0.018	0.036	0.100	0.135	0.023	0.049	0.018	0.032	0.026	0.042	—	0.074	0.048	—
Skeletal muscle	1.25	1.65	6.60	10.3	0.84	6.19	0.78	1.97	0.26	3.69	<2.34	5.38	7.91	2.21
Bone†	7.44	4.80	1.83	2.32	11.8	7.99	1.18	4.28	12.90‡	5.20	13.3	14.6	6.54	10.0
Brain	0.018	0.014	0.020	0.050	0.050	0.023	0.018	—	0.031	—	0.092	0.019	—	0.030
Skin	0.59	1.35	1.11	1.41	1.68	0.94	0.61	1.44	1.92	<0.01	—	2.60	1.80	—
Injected ear	0.24	7.43	0.12	0.14	0.60	0.18	3.27	1.12	0.31	0.42	7.36	0.87	1.60	6.97
Excreted, urine	0.49	none	none	0.81	0.15	5.92	0.69	0.22	1.16	6.99	15.4	1.39	2.52	4.69
,, faeces	none	none	none	none	0.02	0.75	0.55	0.08	0.75	1.39	2.3	0.80	none	1.01
Total recovery	103%	95%	76%	90%	98%	104%	77%	93%	82%	74%	102%	85%	94%	90%

* Pregnant.
† Based on concentration in diaphyses. Excluding marrow.
‡ Based on half concentration in epiphyses. Excluding marrow.
§ Died 22 hr. 40 min. after injection of lead acetate.
‖ The injected solution contained some particulate matter, presumably basic lead acetate.

between different rabbits killed at the same time: 50–70 per cent of the entire dose was found in the liver, where, as in the bone marrow and the spleen, the highest concentrations of lead (10–50 μg./g.) occurred. Concentrations of 1–5 μg./g. occurred in the red cells, lungs, kidneys, and bone. All the other tissues regularly sampled contained about 0.05 to 0.5 μg. of lead per gramme. A small amount of lead was excreted in the urine (Fig. 1), less than 1 per cent in twenty-four hours: and to judge from the movement of lead in the gut contents, another 1 per cent or so was likely to be excreted in the faeces on the second and third days.

When BAL was administered several differences in the distribution of lead were observed. The content and concentration of lead in the liver, spleen, and bone marrow were consistently reduced, except in the liver at six hours. Data at six hours have been obtained for only one normal and one BAL-treated rabbit; the closely similar concentrations found in the livers do not seem to be an outstanding exception to the thesis that BAL reduces the amount of lead in the liver. The concentration of lead in red cells was also consistently lower after BAL, but this effect was offset by an increase in the amount in the plasma, so that the effect on total blood lead was variable. On the other hand, the excretion of lead in the urine was increased five- or tenfold (Fig. 1 and Table III). The increase was still apparent between twelve and twenty-four hours even when BAL had not been given after the fifth hour. In view of the usually transient effects of BAL on the excretion of other metals (Wexler, Eagle, Tatum, Magnuson, and Watson, 1946; Eagle, Magnuson, and Fleischman, 1946; Eagle, Germuth, Magnuson, and Fleischman, 1947) this finding was somewhat unexpected. In the rabbit which died at twenty-three hours (No. 93)

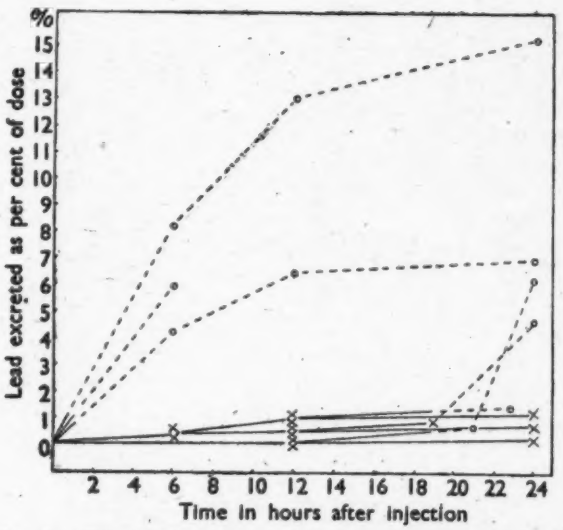


FIG. 1.—The effect of BAL on the cumulative excretion of lead in the urine of rabbits after the intravenous administration of lead acetate (2.07 mg. Pb/kg.).

Ordinates: amount of lead excreted as a percentage of the dose administered (1% = 20.7 μg. Pb/kg. body weight). Abscissae: time in hours after injection of lead. BAL given intramuscularly, either 50 mg./kg. at 1 hour and 12.5 mg./kg. at 5 hours, or 50 mg./kg. at 19 hours and 12.5 mg./kg. at 23 hours.

×——× No BAL or before BAL.
O- - - - - O After BAL.

TABLE III

THE URINARY EXCRETION OF LEAD AFTER INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2.07 MG. Pb/KG.) TO RABBITS, WITH AND WITHOUT SUBSEQUENT INTRAMUSCULAR INJECTIONS OF BAL

Rabbit No.	BAL 50 mg./kg. at (hrs. after lead)	BAL 12.5 mg./kg. at (hrs. after lead)	Urine volume ml. at hours after injection of lead 0–6	6–12	12–18	18–24	Urine lead concentration μg./ml. at hours after injection of lead 0–6	6–12	12–18	18–24
83	nil	nil	50	—	—	—	0.06	—	—	—
86	nil	nil	50	60	60		0.15	0.14	0.07	
90	nil	nil	0	50	50		—	0.04	0.05	
91	nil	nil	18	14	15		0.36	0.91	0.24	
93	19	—	24	18		50*	0.39	0.67		0.29*
94	19	23	25		30†	10‡	<0.03		0.70†	4.35‡
97	19	23	20	28	12	18	0.31	0.20	0.93	5.77
82	1	5	12	—	—	—	10.7	—	—	—
92	1	5	10	5	20		10.0	10.7	0.64	
95	1	5	20	24	36		12.8	6.4	1.90	

The heavy black lines in the body of the table separate values before injection of BAL from values after its injection.
* 18 hours–22 hours 40 minutes after injection of lead acetate.
† 12–21 hours after injection of lead acetate.
‡ 21–24 hours after injection of lead acetate.

the one dose of BAL failed to increase the excretion of lead in the urine. Excretion into the alimentary canal was also greatly increased, but the data are insufficient to show whether the increase was primarily due to biliary or to intestinal excretion. Precautions were not taken to prevent coprophagy, and it is possible that some or all of the lead in the stomach contents got there in that way. Increased concentrations of lead were regularly found in the skeletal and cardiac muscle and in the stomach wall. In the wall of the intestine increased amounts of lead were found in animals killed shortly after administration of BAL, but in the rabbits (Nos. 92 and 95) to which BAL was given at one and five hours and which were killed at twenty-four, any increase produced by BAL had disappeared. In other tissues changes were small or variable. For example, bone diaphysis and lungs showed a decrease in lead content at one hour and an increase at twenty-four hours. In rabbit No. 94 the injected solution was observed to be cloudy, and it appears likely that the exceptionally high concentration in the lungs was due to retention of some particulate matter, probably basic lead acetate, rather than to the action of BAL.

The various changes have been summarized in Table IV, in which tissues having common physiological functions and similar concentrations of lead, and in which the lead concentrations were similarly affected by BAL, have been grouped together. The oversimplifications involved are perhaps justified by the more immediate comprehensibility of the data so presented.

TABLE IV

Summary table of the distribution of lead after intravenous administration of lead acetate (2.07 mg. Pb/kg) to rabbits with and without subsequent intramuscular injections of BAL. The figures given are the mean percentages of the dose found in each group of tissues for all similarly treated rabbits. Details of treatment are as shown in Tables I, II, and III.

Distribution at:	1 hour		6 hours		24 hours		
Treatment:	No BAL	BAL	No BAL	BAL	No BAL	Early BAL	Late BAL
No. of Rabbits:	2	2	1	1		2	3
Liver, spleen, and bone marrow	80	45	76	75	68	52	54
Alimentary canal and contents, faeces, and bile	0.5	14	0.7	4.5	3	7	8
Urine	–	–	0.1	6	0.7	11	3
Blood	4	7	3	1	2	1	1
Skeletal muscle	1.5	8	1	6	1	3	5
Bone	6	2	12	8	7	9	10

Discussion

The extensive literature on the distribution of lead in animals contains few observations on the immediate fate of a single dose of a soluble inorganic salt administered intravenously. The most comprehensive data appear to be those of Weyrauch (1931) and of Kehoe and Thamann (1933) for rabbits and of Behrens (1925) for mice and cats. In general the present results are consistent with those already published (Table V) for the rabbit, at least in order

TABLE V

Distribution of lead one hour after intravascular injection of lead salts (after various authors). The figures given have been deduced from the published data in accordance with assumptions used elsewhere in this paper. Kehoe and Thamann's data are taken from rabbits killed half or two hours after the injection of lead.

	Per cent of dose in various tissues				
Author:	Behrens (1925)			Kehoe and Thamann (1933)	Ginsburg and Weatherall (1948)
Species:	Mouse	Cat	Cat	Rabbit	Rabbit
Lead salt:	Chloride	Chloride	Chloride	Chloride	Acetate
Approx. dose: (mg. Pb/kg.)	15	4	0.04	7–17	2
Blood	20	35	25	2.5	3.5
Spleen	—	—	0.2	2.5	1.5
Liver	29	18	20	8.5	62
Intestine	11	3	6	5	0.5
Kidneys	9	1.5	4	1.5	1
Lungs	—	—	0.6	—	0.9
Heart	0.7	2*	0.4*	—	0.03
Muscle	—	—	5	6.5	1.5
Bone	10	20	20	6.5	6
Brain	—	—	—	1	0.02

*Lead given by intracardiac injection.

of magnitude. However, certain discrepancies are larger than might be expected from differences in dosage and in experimental technique. Except for a single figure of Behrens's for cats (*loc. cit.*, p. 353), bone and marrow apparently have been taken together by previous authors, in unspecified proportion and usually from unspecified sites. In the experiments reported here, bone marrow has been found to contain up to eight times the amount or twenty times the concentration of lead in bone, particularly when relatively marrow-free diaphyses were sampled. It follows that estimates for bone and marrow taken together mean little, as small changes in the proportions of the two tissues in a sample must greatly influence the result. Differences in the lead content of different bones and different

parts of the same bone in acute experiments are partly explicable on this basis. Behrens's one figure for the lead concentration in the bone marrow of a cat is considerably lower than that given for bone in the same animal. This finding is the opposite of all the present observations, and apart from the obvious possibility of a species difference no explanation is apparent. The present findings are also directly the opposite of those described in chronic poisoning in cats, and perhaps rabbits, by Aub, Fairhall, Minot, and Reznikoff (1925). In other tissues the present values for blood and for the alimentary canal are low when compared with the general trend of results. Weyrauch's data are given for dry weights of tissue: suitably amended they appear quite consistent with those here. Kehoe and Thamann's recoveries in their five experiments with lead chloride lasting less than twenty-four hours average 44.5 per cent, and while they have admittedly discarded their "remainder" it is difficult to account for up to (in one case) 88 per cent of the dose outside the liver, spleen, kidneys, washed intestinal tract, blood, muscle, bone, and central nervous system. It seems more probable that losses occurred during administration or estimation, and consequently that their values err towards the low side. Some figures of Brady (quoted by Aub *et al.*, 1925) for the lead content of the livers of anaesthetized rabbits with cannulated bile ducts after considerably larger doses of lead show, like the present data, about 50 per cent or more uptake of lead by that organ in the first few hours after intravenous administration. Behrens's figures indicate that in mice the uptake and excretion of lead by the kidney is much more rapid than in rabbits, and that excretion through the alimentary canal is also much greater: the concentration per unit dose remaining in the tissues is correspondingly less. Urinary excretion in the present experiments has been fairly steady, amounting to not more than 1 per cent of the total dose in twenty-four hours. The early disappearance of lead from the plasma agrees with the observations of Bambach, Kehoe, and Logan (1942), and with those of Mortensen and Kellogg (1944) in dogs and guinea-pigs.

It should be noted that the present work was done with young rabbits. Kasahara and Arimichi (1934) found higher concentrations of lead in the blood of rabbits weighing about 1.0 kg. than in rabbits of double this weight fed on the same relative amounts of lead. Their data can be interpreted as due to differences either in the rate of absorption or of deposition and excretion, but they indicate some difference in the metabolism of lead by young and old animals. It should also be noted that the amount of radioactive material used in the present experiments was large. The possibility exists that some injury was done to those tissues in which the material was most highly concentrated, and that the affinity of the tissues for lead was consequently altered, but it seems unlikely that such an effect would be produced in the short duration of these experiments.

There is at present very little indication how BAL produces the described changes in the distribution of lead. Some of the changes are probably due to the actions of BAL on the rabbit. In view of the cardiovascular effects of BAL (Chenoweth, 1946), it is not surprising to find an increased amount of lead in the splanchnic area after simultaneously administered BAL. But such actions are likely to be less important when lead has been fixed by the tissues, as appears largely to have happened within an hour. Changes observed after this time more probably depend on the relative dissociability of the compounds formed between lead and the tissues and between lead and BAL, on the behaviour of the undissociated lead-BAL compound or compounds, and on the amount of BAL present in a given tissue. The importance or otherwise of the last factor is illustrated by considering the data of Peters, Spray, Stocken, Collie, Grace, and Wheatley (1947), who showed that in a rat one hour after a dose of 100 mg./kg. of BAL containing S^{35}, 2.8 per cent of the dose was found in the liver and 0.17 per cent in the brain. If a similar distribution of BAL is assumed in rabbits poisoned with lead, the amount of BAL in the liver of the rabbits described here would not have greatly exceeded one molecule per atom of lead, even if all the S^{35} were present as unaltered BAL, whereas the amount of BAL in the brain would have been a two-hundredfold excess. In spite of this, BAL had a greater effect on the lead content of the liver than on that of the brain. Without data about the distribution of BAL in the conditions of the present experiments, detailed consideration on these lines is not profitable.

Weatherall (1948) has shown that, after a single dose of lead acetate by stomach tube in rabbits, BAL diminishes the subsequent anaemia, increases the coproporphyrin excretion, and does not greatly affect the mortality. The present observations are consistent with Weatherall's hypothesis that BAL acts by preventing the uptake of lead ions by red cells, and they indicate moreover that BAL can probably remove lead from cells which have already taken it up. On the other hand, no light is thrown on the increased coproporphyrin excretion, nor on the increased mortality produced by BAL in subacute lead poisoned rabbits (Braun, Lusky, and

Calvery, 1946), nor on the prevention of the renal effects of lead in rats (Chiodi and Sammartino, 1947). Further and more chronic experiments are necessary to provide evidence on these points.

Summary

1. The distribution of lead in the tissues of rabbits after intravenous injection of lead acetate (2.07 mg. Pb/kg.) has been studied by the use of the short-lived isotope Pb^{212} (thorium B).

2. The distribution has been similar in most respects at 1, 6, and 24 hours. High concentrations were found in the liver, spleen, and bone marrow: moderate concentrations in red cells, lungs, kidney, and bone: and small amounts in all other tissues: 50–70 per cent of the administered lead was found in the liver, 15–25 per cent in bone marrow, and 5–12 per cent in bone. Less than 1 per cent was excreted in the urine in the first 24 hours, and 1–2 per cent was found in the contents of the gut.

3. After intramuscular injection of BAL the urinary excretion of lead was increased to 3–15 per cent of the dose, even when BAL was not given until nineteen hours after the lead, and larger amounts of lead were found also in the contents of the gut.

4. Other changes when BAL was administered were a reduction of the concentration of lead in the liver, spleen, bone marrow, and red cells, and an increase of the concentration in cardiac and skeletal muscle. After BAL 20–48 per cent (in one case 60 per cent) of the lead was found in the liver, 7–13 per cent (in one case 30 per cent) in the bone marrow, 1–13 per cent in bone, and up to 10 per cent in skeletal muscle.

5. The significance of these changes is discussed.

We wish to thank Miss Irene Munro and Miss Margaret Thomson for technical assistance; various members of the Department of Natural Philosophy, Edinburgh University, for their co-operation; and particularly Dr. E. Broda for much help in developing the technique used in these experiments. We are grateful also to the Therapeutic Research Corporation for a personal grant to one of us (M.G.) and to the Medical Research Council for an expenses grant to the other of us (M.W.).

References

Aub, J. C., Fairhall, L. T., Minot, A. S., and Reznikoff, P. (1925). *Lead Poisoning*, Baltimore: Williams and Wilkins.

Bambach, K., Kehoe, R. A., and Logan, M. A. (1942). *J. Pharmacol.*, **76**, 326.

Behrens, B. (1925). *Arch. exp. Path. Pharmak.*, **109**, 332.

Braun, H. A., Lusky, L. M., and Calvery, H. O. (1946). *J. Pharmacol.*, **87**, supplement, 119.

Chenoweth, M. B. (1946). *J. Pharmacol.*, **87**, supplement, 41.

Chiodi, H., and Sammartino, R. A. (1947). *Nature*, London, **160**, 680.

Eagle, H., Germuth, F. G., Jr., Magnuson, H. J., and Fleischman, R. (1947). *J. Pharmacol.*, **89**, 196.

Eagle, H., Magnuson, H. J., and Fleischman, R. (1946). *J. clin. Invest.*, **25**, 451.

Kasahara, M., and Arimichi, K. (1934). *Z. ges. exp. Med.*, **92**, 629.

Kehoe, R. A., and Thamann, F. (1933). *J. Lab. clin. Med.*, **19**, 178.

Levine, C. J., Mann, W., Hodge, H. C., Ariel, I., and Du Pont, O. (1941). *Proc. Soc. exp. Biol.*, N.Y., **47**, 318.

Mortensen, R. A., and Kellogg, K. E. (1944). *J. cell. comp. Physiol.*, **23**, 11.

Nye, R. N. (1931). *Proc. Soc. exp. Biol.*, N.Y., **29**, 34.

Peters, R. A., Spray, G. H., Stocken, L. A., Collie, C. H., Grace, M. A., and Wheatley, G. A. (1947). *Biochem. J.*, **41**, 370.

Weatherall, M. (1948). *Brit. J. Pharmacol.*, **3**, 137.

Wexler, J., Eagle, H., Tatum, H. J., Magnuson, H. J., and Watson, E. B. (1946). *J. clin. Invest.*, **25**, 467.

Weyrauch, F. (1931). *Z. ges. exp. Med.*, **75**, 706.

Brit. J. Pharmacol. (1948), **3**, 231.

THE EFFECT ON GASTRIC SECRETION OF DIFFERENT RATES OF HISTAMINE INFUSION AND OF "NEOANTERGAN"

BY

D. R. WOOD

From the Department of Pharmacology, Oxford, and the Department of Pharmacology and Therapeutics, Sheffield

(Received January 1, 1948)

In acute experiments on lightly anaesthetized cats, a quantitative study has been made of the gastric secretory response to continuous intravenous infusion of histamine solutions. This is a necessary preliminary to the use of the method in the investigation of the effects of various substances on gastric secretion. The use of an infusion of a low concentration of histamine as a gastric secretory stimulant is not new. Ivy and Javois (1924), Gutowski (1924), Teorell (1932, 1933), Emmelin *et al.* (1941), Uvnäs (1943), Emmelin (1945), Emmelin and Frost (1947), and Öbrink (1946) have reported experiments using this method. However, the earlier workers did not maintain a constant rate of infusion for more than a short period. Emmelin (1945) used a "special device" and Öbrink (1946) used the apparatus described by Lindgren (1943) for the continuous delivery at a constant rate of small volumes of solution from a syringe. The apparatus used in the present work is described below.

Information was sought on the possibility of maintaining, for some hours, a steady output of gastric juice and acid in the anaesthetized cat. It was also desired to find out what rate of histamine infusion would give a submaximal stimulation of the secretory cells, such as would be necessary if the method were to be useful for the investigation or assay of inhibitory substances. Some guidance was given by the results of previous workers. Figures for infusion rates have been calculated approximately from data given in their publications and are presented for convenience in Table I. The rate of infusion is given in terms of μg. of histamine base per kg. of body weight per minute. Where the weight of the animal was not recorded (marked *), average weights of 10 and 3 kg. have been arbitrarily assumed for dog and cat respectively.

TABLE I

Source	Animal	Anaesthetic	Infusion μg./kg./min.	Remarks
Gutowski (1924)	dog*	decerebrate	2	
Ivy and Javois (1924)	pouch dog	none	2.7	submaximal (30–45 min.)
Teorell (1933)	cat*	chloralose and urethane	2.2 (approx.	submaximal (15 min.)
Emmelin *et al.* (1941)	cat	decerebrate	0.5–1.5	
	pouch dog	none	0.5–0.6	
Björkman *et al.* (1943)	cat	chloralose and urethane	5.1	maximal
			1.7	submaximal
Öbrink (1946)	pouch dog	none	2.2	maximal
Emmelin and Frost (1947)	cat	chloralose	2–7 usually 2–3	

From all these reports it seemed likely that an infusion rate of about 2 to 3 μg. histamine base per kg. of body weight per minute would stimulate a submaximal gastric secretion in most anaesthetized cats.

The part played by histamine as a natural gastric secretory stimulant under normal and pathological conditions in man is still undecided; however, the fact that histamine can stimulate gastric secretion has led many workers to investigate the action of antihistamine substances on this response to histamine. Such substances might be useful in the investigation of the exact role of histamine in normal gastric secretion, apart from a possible therapeutic application. There have been conflicting reports on the effects of such substances on gastric secretion, and the position has recently been summarized in the review by Loew (1947). Present

opinion (Grossman and Ivy, 1946; Loew, 1947) is that none of the antihistamine substances so far tested has any significant inhibitory effect on histamine-induced gastric secretion.

The potent antihistamine substance, β-dimethyl-aminoethyl-N-*p*-methoxybenzyl-α-aminopyridine ("neoantergan," 2786 R.P.), has recently been introduced in this country as "anthisan." Bovet and Walthert (1944) reported that it did not inhibit gastric secretion induced by histamine in the rat, and this was confirmed in man by Decourt (1945).

Some experiments have been made, using the method described below, to study the effect of neoantergan on the gastric secretory effect of histamine in the anaesthetized cat.

Experimental Methods

Cats weighing from 1.75 to 4.72 kg. (average, 2.67 kg.) were used after preliminary withholding of solid food for 18 to 24 hours. In a few animals anaesthesia was induced with chloroform and continued with cyclopropane and oxygen. The depth of anaesthesia could be readily varied from complete surgical anaesthesia during the operative procedure to a light plane of narcosis during the remainder of the experiment. The main disadvantage apart from the slight danger of explosion is that of expense. In most animals anaesthesia was produced by intraperitoneal injection of sodium pentobarbitone, small maintenance doses being given intravenously when required later. The anaesthetized animal was prepared for collection of gastric secretion by the method of Lim (1923) as recently modified by Roth and Ivy (1944).

The stomach and duodenum are approached by a midline abdominal incision and a cannula is inserted into the stomach through the pylorus from an opening in the first part of the duodenum. The duodenum is ligated just proximal to the entry of the bile duct and the cardio-oesophageal junction is also tied, the vagi being excluded from the ligature. The cannula is a piece of perforated soft rubber tubing about 7 cm. long and of about 0.4 cm. bore. It is tied on to a short length of glass tubing around which the pyloric ligature is tied, the local blood vessels being carefully preserved. From the other end of the glass tubing another short rubber tube passes to the exterior through a stab wound in the right side of the abdominal wall, at the level of the pylorus. The animal is arranged slightly tilted on its right side so that the gastric juice flows readily from the stomach, without stasis. In only two of some seventy animals have more than a few drops (<0.5 ml.) been found in the stomach at the end of 6–7 hours of collection of juice. The stomach is gently rinsed out with warm saline via the pyloric cannula, and after closure of the abdominal incision the animal is left for an hour before the infusion is commenced. The whole procedure takes only a few minutes, and under the conditions described the rate of secretion at the end of one hour is basal and of the order of one or two drops in ten minutes, no free acid being detected. It is important that the animal be kept warm during the whole of the experiment.

The infusion apparatus was designed and made by Dr. E. H. J. Schuster; it incorporates a cylinder with close-fitting reversible piston, originally a small hydraulic jack. This apparatus delivers a constant slow flow of liquid paraffin to a vessel from which the infusion fluid is displaced into the cat. By means of an electric gramophone motor and a variable reversible gear, a continuous infusion can be maintained indefinitely at a rate variable from 0.19 to 6.0 ml. per min. Histamine acid phosphate is made up in normal saline, and the infusion of 0.75 ml. of saline per min. easily replaces the fluid and chloride lost in the gastric juice. (Throughout this paper doses of histamine are doses of the base.) In control animals infusion of saline alone did not cause any secretion greater than the basal rate. The total volume of juice and the amounts of free and total acid were measured, samples being collected at intervals of 10 to 30 minutes for up to 7 hours. For acid titration *N*/50 or *N*/20 sodium hydroxide was used with thymol blue as the usual indicator for the two endpoints. Peptic activity of the juice has not been measured.

In most of the experiments with neoantergan it was infused with histamine in the same solution. In three other experiments, after an initial period of two hours during which 5 μg. histamine per min. was infused, the infusion was rapidly changed to a mixture of 5 μg. histamine and 15 μg. neoantergan per min. for 1½ to 2 hours. Finally the original solution of histamine was substituted for the mixture. The solutions were changed with only a momentary interruption of the flow since it was arranged that the pump could deliver liquid paraffin to either of two bottles by changing a number of clips.

Results

The average volumes of juice and of free acid secreted by groups of 6 or 7 cats during successive 30-min. periods of histamine infusion are shown in Fig. 1. The four groups of cats received histamine infusions of 2.5, 5, 10, and 20 μg. per min. respectively for 5–6 hours. After an initial delay varying from about 30 to 90 minutes, during which there is a steady increase in secretion, a reasonably constant rate of secretion is maintained for some 5 hours at least. The initial delay is less at the higher rates of infusion, as might be expected. The groups receiving 10 and 20 μg. histamine per min. achieve much the same peak rate of secretion. When the average hourly secretion rates are compared with the rate of infusion as in Table II and Fig. 2, it can be seen that although there is a wide variation between the responses of individual cats there is a relation between the mean rate of secretion in a group of cats and the rate of histamine infusion. It is certain that in most cats secretion is not maximally stimulated by an infusion of 5 μg. histamine per min. and probably not by 10 μg. per min. If the rates

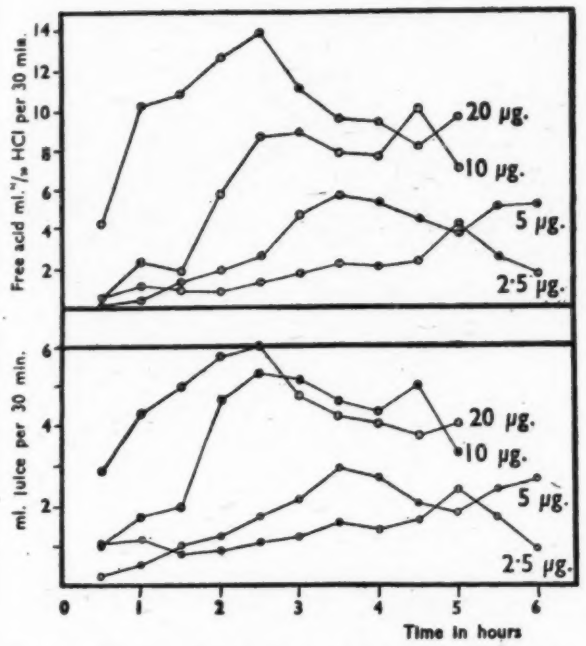


FIG. 1.—Mean volumes of juice and free acid secreted in successive 30-minute periods by groups of cats receiving different rates of histamine infusion. 2.5, 5, 10, and 20 μg./min. (equivalent to range of 0.74 to 1.25, 1.4 to 2.3, 3.4 to 4.5, and 5.3 to 9.8 μg. histamine/kg./min.) in groups of 6, 7, 6, and 6 cats respectively. Infusion started at zero time. Abscissae, time in hours. Ordinates ml., juice and *N*/20 HCl in 30 min.

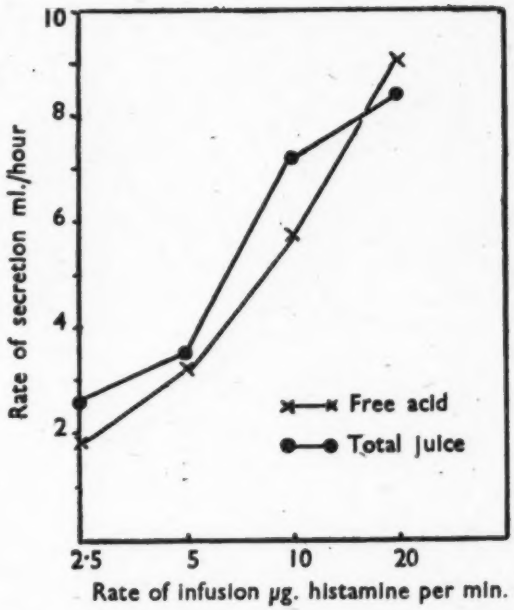


FIG. 2.—Average hourly secretion rates (gastric juice and free acid) over a period of 5–6 hours in groups of cats, at different rates of histamine infusion. Abscissae: rate of histamine infusion (logarithmic. scale). Ordinates: rate of secretion.

of infusion are correlated with the weights of the individual animals it is found that a rate of histamine infusion in excess of 4 μg. per kg. per min. causes maximal secretion in most animals. The secretion due to 2 to 3 μg. histamine per kg. per min. is usually submaximal.

Infusion of neoantergan alone (15 μg./min.) did not stimulate gastric secretion in excess of the "basal" level and no free acid was detected in such juice. In Figs. 3 and 4 average secretion curves are compared for groups of cats given histamine alone and mixtures of histamine and neoantergan. Secretion in a group of cats given 5 μg. histamine and 15 μg. neoantergan per min. is greater than that due to 5 μg. histamine per min. alone (Fig. 3). This is also apparent from the average hourly secretion rates shown in Table III. From Fig. 4 it will be seen that

TABLE II

MEAN RATES OF SECRETION OF JUICE AND FREE ACID IN RELATION TO RATE OF HISTAMINE INFUSION

(Histamine doses are given in terms of free base; the acid phosphate was used)

Group rate of infusion	No. of cats	Avge. wt. (kg.)	Range of individual infusion rates μg./kg./min.	Mean rate of secretion of juice			Mean rate of acid production (*N*/10 HCl)		
				ml./hr.	σ	ϵ	ml./hr.	σ	ϵ
2.5 μg./min. ..	6	2.7	0.74–1.25	2.61	1.78	0.73	1.89	2.24	1.00
5.0 μg./min. ..	7	2.85	1.4 –2.3	3.50	2.25	0.85	3.26	3.33	1.26
10.0 μg./min. ..	6	2.52	3.4 –4.5	7.20	3.50	1.43	5.78	3.19	1.30
20.0 μg./min. ..	6	2.58	5.3 –9.8	8.42	2.41	0.98	9.07	4.47	1.83

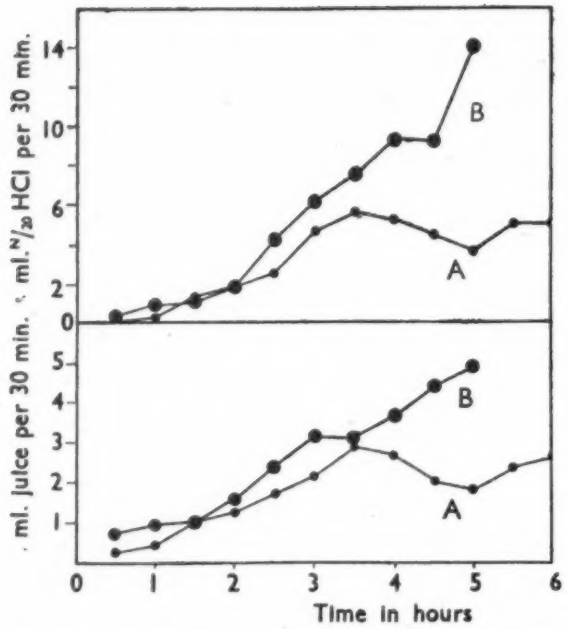


FIG. 3.—Effect of neoantergan. Comparison of secretion of gastric juice and free acid due to 5 μg. histamine/min. (A) and a mixture of 15 μg. neoantergan and 5 μg. histamine/min. (B). Secretion induced by the mixture is greater than with histamine alone. Figures are average values for groups of 7 and 6 cats respectively.

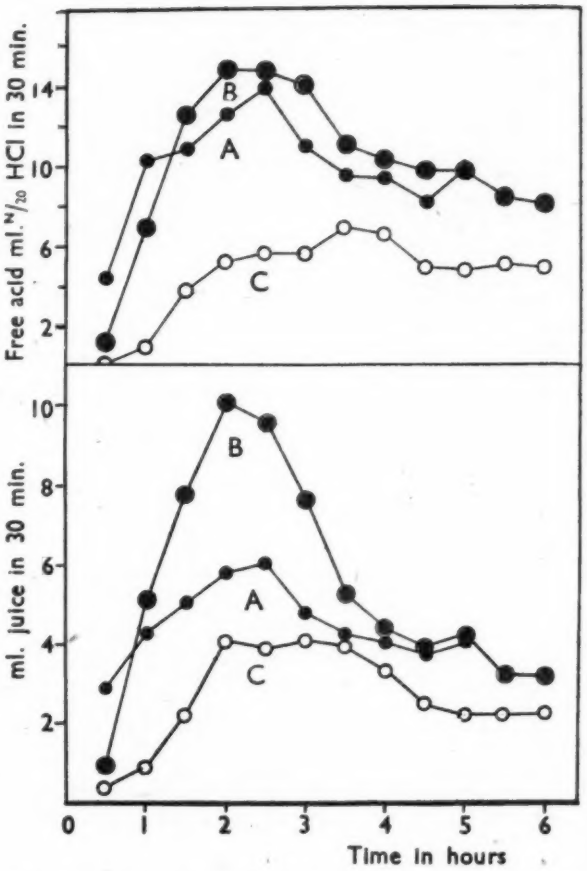


FIG. 4.—Effect of neoantergan. Comparison of average secretion curves for juice and free HCl due to 20 μg. histamine per min. (6 cats) (A) with that due to infusion of the same amount of histamine and 5 or 15 μg. neoantergan per min. (3 cats each). 15 μg. neoantergan (B); 5 μg. neoantergan (C).

TABLE III

THE EFFECT OF NEOANTERGAN ON GASTRIC SECRETION INDUCED BY HISTAMINE INFUSION, 5 μG./MIN.

Cat No.	Weight kg.	Duration, hours	Average rate of secretion: Juice, ml./hr.	Average rate of secretion: N/10 acid, ml./hr.
A. Infusion 5 μg. histamine per min.				
28	2.75	6	5.90	5.85
34	3.25	6	1.20	0.50
35	2.80	5	5.20	6.65
36	2.50	5	0.65	0.00
37	2.90	6	1.90	0.65
45	2.18	5	3.75	3.85
58	3.60	7	5.90	5.30
Mean rate ± standard error			3.50 ± 0.85	3.26 ± 1.26
B. Infusion 5 μg. histamine + 15 μg. neoantergan per min.				
29	2.50	6	3.10	2.50
40	2.45	5	3.95	0.75
43	1.87	4	5.50	5.55
44	1.75	5	6.20	6.85
47	2.50	6	12.05	15.55
48	3.48	5	1.80	0.80
Mean rate ± standard error			5.43 ± 1.47	5.33 ± 2.08

the secretion produced by 20 μg. histamine and 15 μg. neoantergan per min. is probably greater than that due to histamine alone, although the secretion produced by 20 μg. histamine and 5 μg. neoantergan per min. is less than that due to histamine alone. These contrary findings may be partly due to the small number of animals tested in this group (Table IV).

In two of three other experiments, in each of which the effects of infusions of histamine and neoantergan and of histamine alone were compared in the same animal, there was a definite increase in secretion of juice and acid during the period of the mixed infusion compared with that due to histamine only (Fig. 5). The effect was absent in the third cat. Certainly no inhibition of gastric secretion was observed.

TABLE IV

THE EFFECT OF NEOANTERGAN ON GASTRIC SECRETION INDUCED BY 20 μG. HISTAMINE/MIN.

Cat No.	Weight kg.	Duration, hours	Average rate of secretion: Juice, ml./hr.	Average rate of secretion: N/10 HCl, ml./hr.
20 μg. histamine per min.				
15	2.05	4	3.65	1.05
16	2.50	5	9.10	9.80
17	2.30	6	9.20	8.85
18	2.45	6	9.85	10.05
20	2.35	7	10.15	14.80
64	3.80	5	8.50	9.90
Mean rate ± error			8.41 ± 0.99	9.08 ± 1.83
20 μg. histamine and 5 μg. neoantergan per min.				
22	2.50	6	4.35	3.50
23	3.50	6	2.80	1.50
24	2.75	6	8.85	9.05
Mean rate ± error			5.33 ± 1.82	4.67 ± 2.24
20 μg. histamine and 15 μg. neoantergan per min.				
25	1.80	6	11.80	2.15
26	1.80	6	12.90	19.20
27	2.70	6	7.70	8.95
Mean rate ± error			10.8 ± 1.58	10.1 ± 4.95

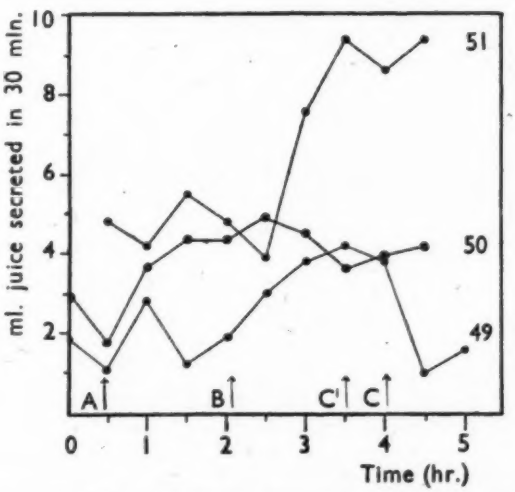


FIG. 5.—Effect of neoantergan on gastric secretion of cats 49, 50, and 51: 5 μg. histamine per min. at A and C (C′ for cat 51); 5 μg. histamine and 15 μg. neoantergan per min. between B and C (C′ for cat 51). Pentobarbitone anaesthesia.

The majority of the results suggest the probability that neoantergan may increase rather than reduce the stimulation of gastric secretion by histamine.

DISCUSSION

Continuous intravenous infusion of histamine causing submaximal secretion of gastric juice and acid has given in most animals a reasonably steady flow of juice and acid for some hours after the first 30 to 90 minutes; during this initial period the secretion is increasing to the steady level. At the highest rates of infusion used there is a tendency for the rate of secretion, which is probably maximal, to fall off after 5–6 hours. In most animals a rate of infusion of 2 to 3 μg. histamine per kg. per min. will not stimulate gastric secretion maximally, and this rate of infusion is probably suitable if it is desired to test the effect of other substances on the secretion produced by histamine. In a particular animal one can test whether the secretion is in fact submaximal by observing the effect of increasing the rate of infusion once a steady rate of secretion has been obtained. The former level of secretion is usually regained within 30 minutes of reducing the infusion rate again.

The effect of substances which antagonize histamine or histamine-induced gastric secretion can be investigated. The test substance may be injected in single doses during the histamine infusion, or average secretion curves for groups of animals receiving histamine alone can be compared with similar curves for groups receiving histamine and the antagonist. A quantitative estimation of the effect can be obtained with either method.

The finding that neoantergan did not inhibit the effect of histamine on gastric secretion was expected in view of earlier work. There seems little doubt that, like other less potent antihistamine agents, neoantergan does not decrease the gastric secretory effect of histamine. Even the much more potent thiodiphenylamine derivative, 3277 R.P., has been shown to be ineffective against the gastric ulceration produced by large doses of histamine in the guinea-pig, although protecting the animal from the immediately lethal effect of the histamine (Halpern and Martin, 1946). Clinical evidence confirms that neoantergan has no value in the control of gastric hypersecretion (Decourt, 1945). The evidence reported here strongly suggests that neoantergan may actually increase the gastric secretory response to injected histamine. Similar effects after the administration of benadryl have been reported by Emmelin and Frost (1947) in anaesthetized cats and by McElin and Horton (1946) and Doran (1947) in man.

This probable potentiation of histamine action on gastric secretion may link up with the reported aggravation of symptoms in some asthmatic patients treated with antihistamine substances (see Bovet and

Walthert, 1944, p. 38). These authors pointed out that this finding might be due to an increased "histaminaemia," and Geiringer (1947) has recently reiterated this possibility in a comment on the conclusions of Doran. Proof of such an explanation of the apparently anomalous finding awaits further experiment.

Summary

1. Gastric secretion has been collected from cats, anaesthetized with sodium pentobarbitone, by a cannula tied into the stomach through the pylorus. Volumes of juice and of free and total acid secreted have been measured.

2. The mean rate of secretion of juice and of acid in a group of cats is related to the rate of infusion of histamine, although the rate of secretion obtained with a certain rate of histamine infusion varies considerably from animal to animal.

3. The infusion of 2 to 3 μg. histamine per kg. per min. causes a submaximal secretion in most cats, which remains relatively steady after the first 30 to 90 minutes for at least 5 or 6 hours.

4. The method can be used to investigate the effect of other substances on the gastric secretion induced by histamine.

5. The antihistamine substance neoantergan has been found to increase rather than to inhibit the gastric secretion induced by an infusion of histamine. The implications of this finding are discussed.

These experiments were begun in the Department of Pharmacology, Oxford, at the suggestion of Prof. J. H. Burn. To him and to Prof. E. J. Wayne I am grateful for continued stimulation and interest. Neoantergan and anthisan were kindly supplied by Dr. D. Bovet and by Dr. R. Wien. Part of the expenses have been met by a grant from the Medical Research Council.

References

Bovet, D., and Walthert, F. (1944). *Annal. pharmaceut. franc.* **2**, suppl. 4.
Decourt, J. (1945). *Sem. Hôp. Paris.*, **21**, 707.
Doran, F. S. A. (1947). *Lancet*, **2**, 490.
Emmelin, N. (1945). *Acta physiol. scand.*, **11**, suppl. 34.
Emmelin, N., and Frost, J. (1947). *Acta physiol. scand.*, **13**, 75.
Emmelin, N., Kahlson, G., and Wicksell, F. (1941). *Acta physiol. scand.*, **2**, 123.
Geiringer, E. (1947). *Lancet*, **2**, 773.
Grossman, M. I., and Ivy, A. C. (1946). *Gastroenterology*, **7**, 134.
Gutowski, B. (1924). *C. R. Soc. Biol., Paris*, **91**, 1346.
Halpern, B. N., and Martin, J. (1946). *C. R. Soc. Biol., Paris*, **140**, 830.
Ivy, A. C., and Javois, A. J. (1924). *Amer. J. Physiol.*, **71**, 604.
Lim, R. K. S. (1923). *Quart. J. exp. Physiol.*, **13**, 71.
Lindgren, G. (1943). *Acta physiol. scand.*, **6**, 286.
Loew, E. R. (1947). *Physiol. Rev.*, **27**, 542.
McElin, T. W., and Horton, B. T. (1946). *Gastroenterology*, **7**, 100.
Öbrink, K. J. (1946). *Acta physiol. scand.*, **12**, 213.
Roth, J. A., and Ivy, A. C. (1944). *Amer. J. Physiol.*, **141**, 454.
Teorell, T. (1932). *Pflügers Archiv.*, **231**, 140.
Teorell, T. (1933). *Skand. Arch. f. Physiol.*, **64**, 225.
Uvnäs, B. (1943). *Acta physiol. scand.*, **6**, 97.

Brit. J. Pharmacol. (1948), **3**, 237.

THE PHARMACOLOGICAL PROPERTIES OF CONESSINE, *ISO*CONESSINE AND *NEO*CONESSINE

BY

R. P. STEPHENSON

From the Department of Pharmacology, Oxford

(Received January 19, 1948)

Conessine is an alkaloid obtained from the bark and seeds of *Holarrhena antidysenterica*, a tree growing in India. The alkaloid has been studied by many workers (Keidel, 1878; Burn, 1914; Chopra, Gupta, David and Ghosh, 1927; Chopra, 1933; Bakhsh, 1936) who have described various properties bearing little or no relation to one another. Conessine is a typical example of the many alkaloids described in reference books of pharmacology the properties of which are difficult to remember because there is no sign of connexion between them and because they appear to be entirely fortuitous.

A report from Frère Just. Gillet, S. J., missionary in the Belgian Congo, that the chewing of the leaves of the plant *Holarrhena congolensis* produced anaesthesia of the mucous membrane of the mouth, led Burn (1914) to examine conessine experimentally for local anaesthetic action, which he found it to possess. Further work on this action was carried out by Trevan and Boock (1927). The use of conessine as a local anaesthetic is, however, limited by the fact that when injected it causes necrosis. Two isomers of conessine, namely *iso*conessine and *neo*conessine, have been prepared by Dr. S. Siddiqui by treating conessine with sulphuric acid. Samples of conessine dihydrochloride, *iso*conessine hydrochloride and *neo*conessine hydrochloride were left with Prof. Burn by Dr. Siddiqui with the request that they should be compared with one another for local anaesthetic potency and for local irritant action. There was a possibility that one of these substances would prove as potent as conessine and not cause an inflammatory reaction when injected.

The three substances have therefore been compared for their irritant action, and the method used for making this comparison will be described in a later paper. They have also been compared for local anaesthetic action. In addition these substances have been examined for other properties; several local anaesthetics are known to depress the action of acetylcholine on the rectus muscle of the frog, and de Elío (1948) has even observed a quantitative parallelism. Dawes's work (1946) showed that many local anaesthetics had a quinidine-like action on the heart. Conessine and its isomers have therefore been examined on skeletal and on cardiac muscle. Since the results indicated a similarity to quinidine, and since de Elío (1948) had shown that quinidine had a spasmolytic action, reducing the action of acetylcholine on the intestine, conessine and its isomers have also been tested in this way.

Experimental Observations

Local anaesthetic action.—No figures exist for the relation between the local anaesthetic potency of procaine and conessine when given by injection. Conessine and its derivatives were therefore compared with procaine by intracutaneous injection into guinea-pigs according to the method of Bülbring and Wajda (1945). The results for conessine when compared with *neo*conessine and *iso*conessine on the same animals are shown in Fig. 1, from which it appears that *neo*conessine is the weakest, having 48 per cent of the strength of conessine, and *iso*conessine is intermediate, having 77 per cent of the strength of conessine. A careful comparison of

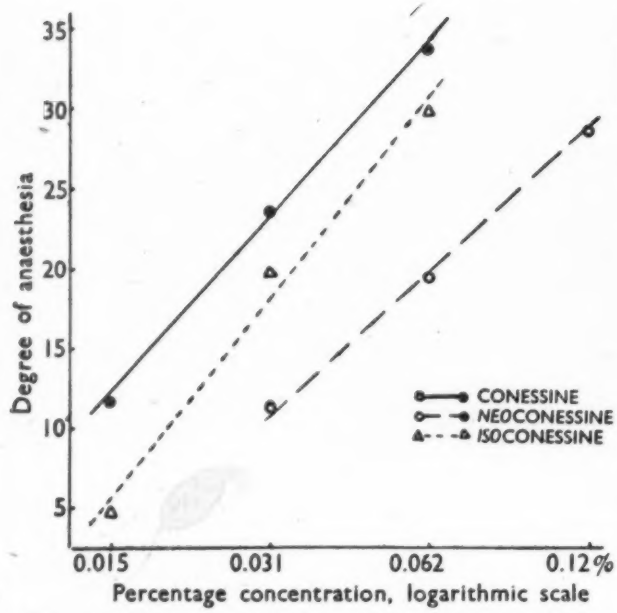


FIG. 1.—Graph showing the relative local anaesthetic potencies of conessine, *iso*conessine and *neo*conessine. Each point represents the mean degree of anaesthesia in 8 areas. If conessine = 100, then *iso*conessine = 77 and *neo*conessine = 48.

TABLE I

LOCAL ANAESTHETIC POTENCIES IN TERMS OF CONESSINE = 100

(Intracutaneous injection into guinea-pigs)

	Local anaesthetic action	Inhibition of ACh on frog rectus
Conessine ..	100	100
*Iso*conessine ..	77	73
*Neo*conessine ..	48	60
Cocaine	47	30
Quinidine ..	10	20
Procaine ..	7	10

conessine with procaine showed that the relative anaesthetic potencies can be expressed as in Table I, in which the figures for quinidine (de Elío, 1948) and for cocaine (Bülbring and Wajda, 1945) are also included for comparison.

Table I shows that conessine is appreciably stronger than its derivatives, but that the weakest, *neo*conessine, differs little in potency from cocaine.

Frog rectus.—The stimulant action of acetylcholine on the frog rectus muscle was found to be depressed by conessine and its isomers. An illustration of this action is given in Fig. 2 in which the effects of all three substances are shown. A direct comparison of *iso*conessine and quinidine showed that quinidine had 28 per cent of the potency of *iso*conessine in depressing acetylcholine on the frog rectus. When de Elío (1948) compared four local anaesthetics, procaine, cocaine, amethocaine and nupercaine, he found that they likewise depressed the action of acetylcholine on the frog rectus and moreover that their relative potency in doing so was similar to their relative potency as local anaesthetics. This was also true for conessine and its isomers as shown in Table I, in which the two sets of figures are placed side by side.

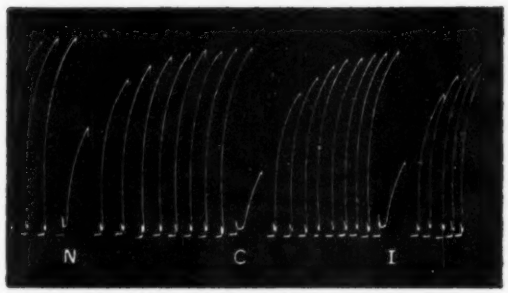


FIG. 2.—Record of contractions of the frog rectus abdominis produced by a concentration of 3.75×10^{-7} acetylcholine acting for 90 sec. The contractions were obtained at 6 min. intervals and those marked N, C and I were preceded by exposure of the muscle for 4½ min. to concentrations of 4×10^{-5} *neo*conessine, conessine and *iso*conessine respectively. If conessine = 100, then *iso*conessine = 73 and *neo*conessine = 60.

Denervated gastrocnemius of cat.—Similar experiments were also carried out on the denervated gastrocnemius muscle of the cat. The left sciatic nerves of a series of cats were divided under ether with aseptic precautions (these operations were kindly performed by Prof. Burn) and the animals were left for 5–26 days. The cats were then anaesthetized with ether and chloralose and the gastrocnemius muscle was detached from the os calcis and fastened to a tension lever. Injections were made through a cannula in the right iliac artery pointing towards the bifurcation of the aorta. Contractions were then obtained by the injection of acetylcholine, and the effect of a preceding injection of conessine on these contractions was determined. An illustration of two experiments is given in Fig. 3. The usual result was that injections from 1–10 mg. depressed the response. In the upper part of Fig. 3 this depression is shown produced by 10 mg. *iso*conessine and by 10 mg. conessine. In the lower part of Fig. 3 a less usual effect is shown, namely, that 1 mg. of these same alkaloids produced an augmentation of the acetylcholine response.

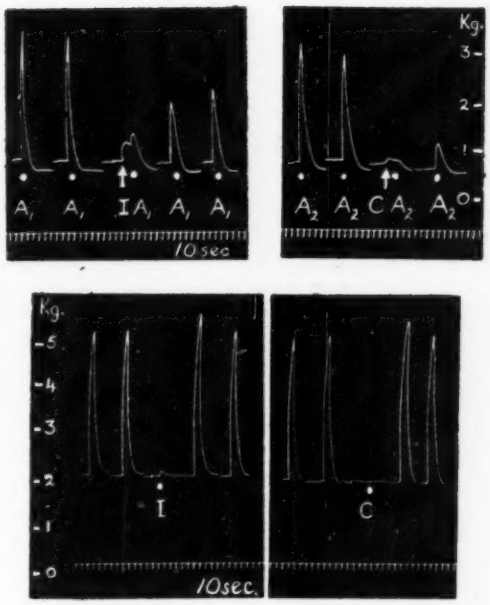


FIG. 3.—Cat. Chloralose. Record of contraction of denervated gastrocnemius in response to intra-arterial injections of acetylcholine.

Upper tracing: Muscle denervated 27 days before. Contractions at A_1 due to 10 μg. ACh. Intra-arterial injection of 10 mg. *iso*conessine produced a small contraction, and the following contraction in response to ACh was greatly reduced. Contraction at A_2 produced by 20 μg. ACh. 10 mg. conessine abolished the effect of the next injection of ACh.

Lower tracing: Muscle denervated 26 days before. Contractions produced by 10 μg. ACh. The intra-arterial injection of 1 mg. *iso*conessine at I, and of 1 mg. conessine at C, slightly increased the effect of the next injections of ACh.

Rat diaphragm preparation.—Since the work of Harvey (1939) it has been known that quinine has various actions on skeletal muscle among which are (1) the ability to increase the tension response to a single maximal stimulus, and (2) to act like curare in lowering the excitability at the motor end plate. Experiments were therefore carried out to see if these effects could be demonstrated when conessine and its isomers were used. It was found that the curare-like action was regularly demonstrable, but only once was the augmentor action seen. The isolated nerve-muscle preparation of the rat as described by Bülbring (1946) was used. The effect is shown in Fig. 4 in which the phrenic nerve was stimulated by maximal single shocks ; in the upper part of the figure the addition of 0.5 mg. *iso*conessine to the bath caused an increase in the response very similar to that seen in the lower part of the figure when 2 mg. quinidine was added. The usual response to the addition of *iso*conessine was

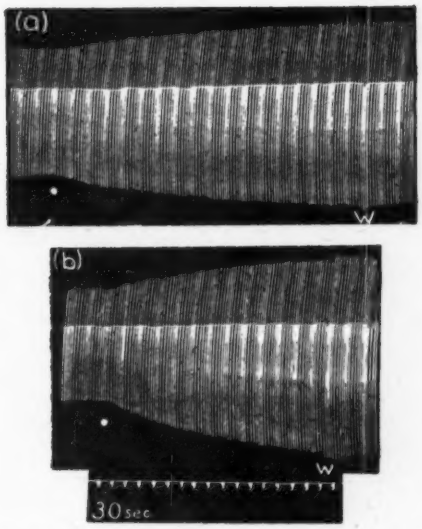


FIG. 4.—Rat diaphragm phrenic nerve preparation. Tyrode 37° C., bath 50 ml. Record of the contractions produced by stimulating the nerve with single maximal shocks 8–10 times per min. At (*a*) is shown the increase in amplitude produced by 0.5 mg. *iso*conessine. This is an unusual effect. At (*b*) is shown the action of 2 mg. quinidine.

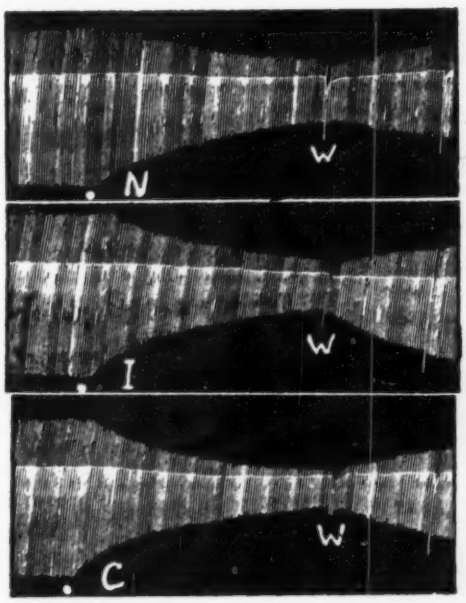


FIG. 5.—Rat diaphragm phrenic nerve preparation. Record of maximal contractions. Depression of contraction produced by 1 mg. *neo*conessine at N, 1 mg. *iso*conessine at I and 1 mg. conessine at C. All three substances were in the bath for 10 min. before washing out. The effects were reversible.

that shown in Fig. 5 in which the muscle twitches were steadily reduced as they are when *d*-tubocurarine is added; the same result was obtained with conessine and *neo*conessine. The doses used were 5–10 times as great as the dose of *d*-tubocurarine which would have been required to produce a similar effect.

Quinidine-like action on cardiac tissue.—The alkaloids were tested by Dawes's method (1946) in which the isolated rabbit auricles are suspended in a bath at 29° C. and driven electrically. The maximum rate at which the auricles will follow the electrical stimulus is determined before, and then after exposure for 10 min. to a given concentration of the substance. When quinidine is tested in this way it reduces the maximum rate, and this reduction persists for some time after the quinidine has been washed out. However, provided an interval is allowed, the preparation returns to its former state and further observations can then be made. When conessine or its isomers were used, a similar reduction in the maximum rate was observed (see Fig. 6), but the effect of a given concentration on different occasions was not the same, and often an increase in concentration produced little increase in effect. From the best experiments it appeared that conessine in a concentration of 2×10^{-5} was approximately equal to quinidine 10^{-5}. Thus the potency of conessine was 50 per cent of that of quinidine. *Iso*conessine was weaker than this.

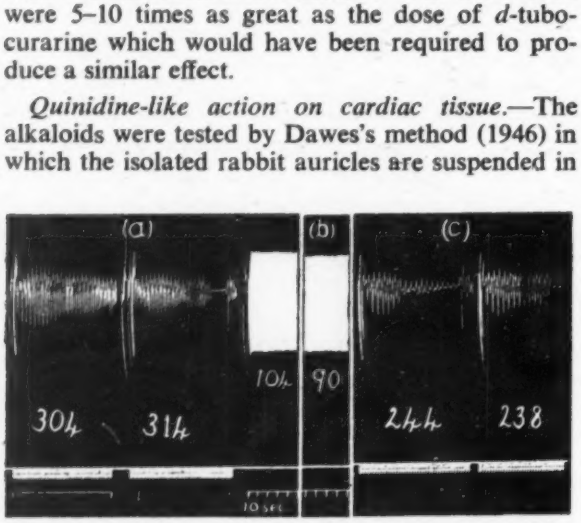


FIG. 6.—Isolated rabbit auricle driven electrically. Ringer-Locke at 29° C., bath 50 ml. (*a*) Auricle follows 304 but does not follow 314 stimuli per min. Spontaneous rate 104 beats per min. (*b*) Spontaneous rate after 5 min. exposure to 4×10^{-5} *iso*conessine. (*c*) After 10 min. exposure to *iso*conessine the auricle fails to follow 244 but follows 238 stimuli per min.

Rabbit auricles.—Acetylcholine causes diminution of the spontaneous contractions of the isolated rabbit auricles, as shown in Fig 7, in which the effect of 50 μg. acetylcholine is shown twice at the beginning of the record. After washing out, when the contractions had recovered their original size, 2 mg. *iso*conessine was added to the bath, and 1 min. later 50 μg. acetylcholine was added again.

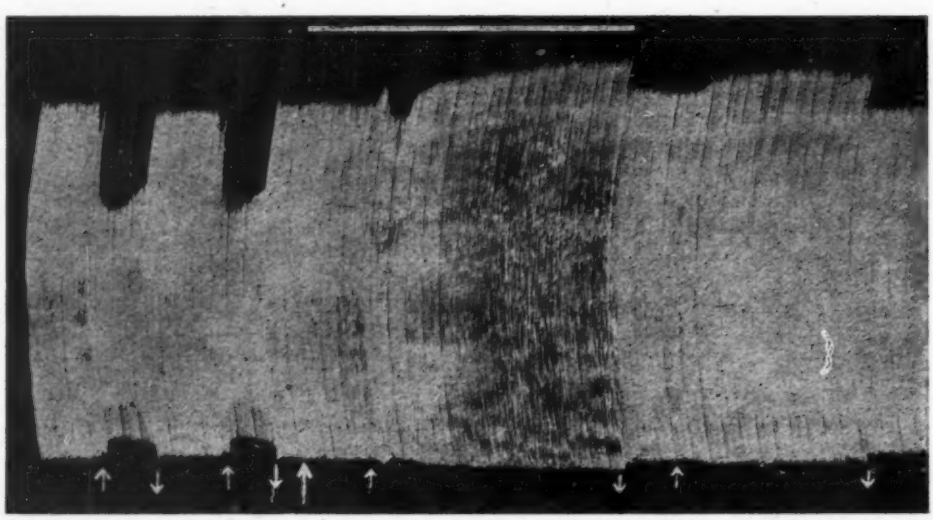


FIG. 7.—Isolated rabbit auricle beating freely. Ringer-Locke at 29° C., bath 50 ml. At the small arrows ↑, 50 μg. ACh was added to the bath and was washed out when the drum was stopped at the reversed arrows ↓. 2 mg. *iso*conessine was added to the bath at the large arrow (1 min. before the next dose of ACh) and remained in the bath during the period shown by the horizontal line. The inhibitory action of ACh was abolished and had not returned 10 min. after washing the *iso*conessine from the bath. The apparent augmentation by ACh in the presence of *iso*conessine was believed to be an artefact.

No diminution was seen, either then, or at a further addition of acetylcholine after the *iso*conessine was washed out. After further washing out, the inhibitory effect of acetylcholine slowly returned, though not fully. Both conessine and *neo*conessine had the same effect.

Action on the heart.—In the rabbit heart perfused by Langendorff's method, the alkaloids were observed to have a dilator action on the coronary vessels which was most marked with *neo*conessine,

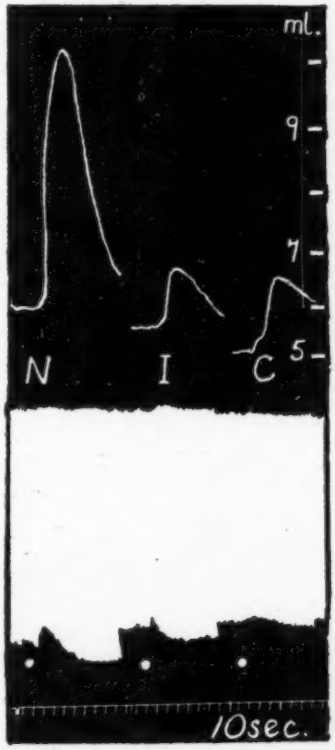


FIG. 8.—Isolated rabbit heart (Langendorff preparation). Perfused with Ringer-Locke at 37° C. Record from above downward: Coronary flow ml. per min., amplitude of heart beats and time marker. At N, I and C 200 μg. of *neo*conessine, *iso*conessine and conessine respectively were injected into the perfusion fluid. Note large dilator effect of *neo*conessine. (For method of recording, see Stephenson, 1948.)

and sometimes absent with conessine. A comparison of the dilator effect of the three substances is shown in Fig. 8, where they are also seen to cause a slight increase in amplitude of the beat. Bakhsh (1936) noted the dilator action of *iso*conessine on the coronary vessels.

Action on blood vessels.—To test the effect on the vessels, both the rat's hindlegs and the rabbit's ear have been used, perfused with Ringer's solution at room temperature. The rat's hindlegs were perfused through the abdominal aorta and the rabbit ear by the method of Gaddum and Kwiatkowski (1938). Conessine, when injected into the fluid perfusing the rabbit's ear, caused vasodilatation. It was curious to find that whereas the constrictor effect of an injection of adrenaline in the rabbit's ear was greatly reduced by an injection of *iso*conessine, the constrictor effect of adrenaline in the rat's hindlegs was not affected. The difference was perhaps more apparent than real, since when an adrenaline tone was maintained in the rat's hindlegs, the injection of conessine (0.1 mg.) caused dilatation (Fig. 9a). In the rat's hindlegs, a single injection of acetylcholine during the maintenance of an adrenaline tone caused dilatation. When not only adrenaline but also conessine was present

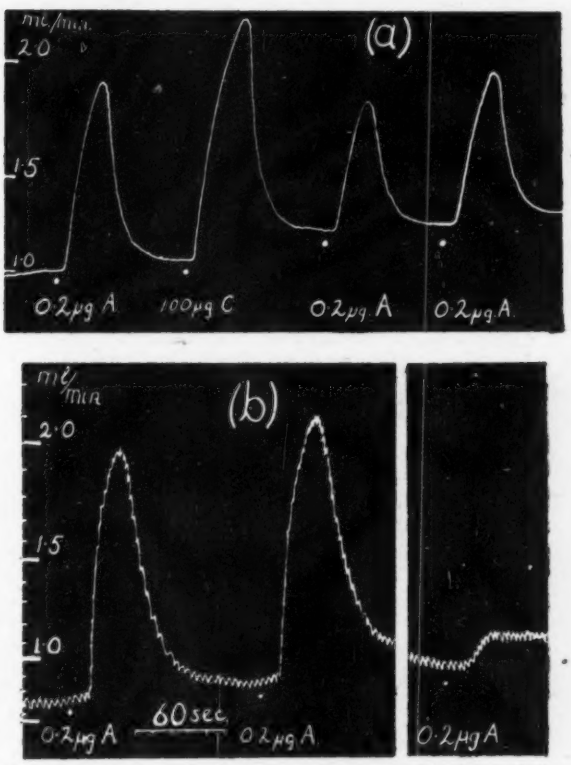


FIG. 9.—(*a*) Record of the flow from a perfusion of the hindlegs of a rat with Ringer-Locke at room temperature. The perfusion contained 10^{-7} adrenaline and dilatations were produced by 0.2 μg. ACh and by 100 μg. conessine. The effect of ACh is reduced after conessine. The record is continuous and the injections were made at 10 min. intervals. (*b*) Dilatations were produced by 0.2 μg. ACh. In the second part the perfusion fluid contained 10^{-5} conessine in addition to adrenaline. Note that, in the presence of conessine, the dilator effect of ACh was greatly reduced.

in the perfusing fluid, the dilator action of acetylcholine was almost abolished (Fig. 9*b*).

Action on blood pressure and respiration.—The actions of *neo*conessine and *iso*conessine were found to be indistinguishable from that of conessine on the blood pressure.

It was shown by Burn (1914) that a large dose of conessine produces a fall of blood pressure in

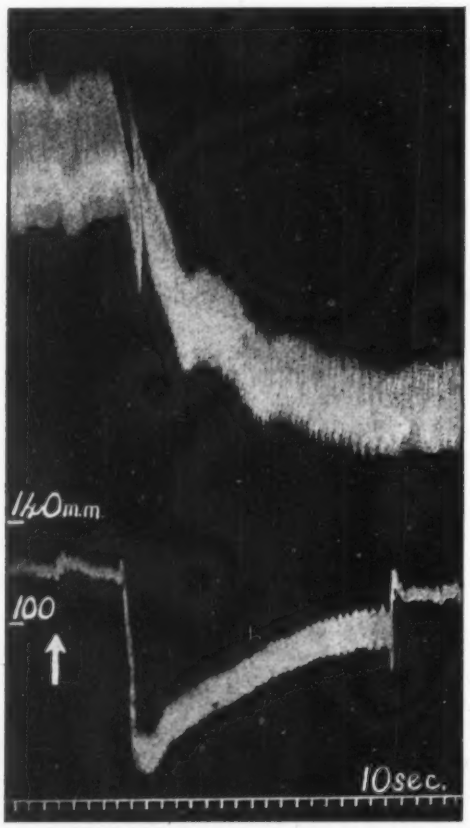


FIG. 10.—Rabbit, urethane. Record of respiration (above) and blood pressure. At the arrow 8 mg. conessine was injected. After about 1 min. this produced a sudden slowing of the heart rate accompanied by a fall of pressure. The pressure slowly recovered and the heart block suddenly disappeared. The respiration was depressed and recovered very slowly and incompletely.

which the heart action at first is unchanged. The fall is presumably due to the vasodilatation which has been described above. After some delay the heart is affected, the ventricular rate becoming very slow; the heart then appears to be in block. When smaller doses are used, either or both of these effects may be seen. In Fig. 10 when 8 mg. conessine was injected there was no initial fall, for vasodilatation did not occur; after a delay of 1 min. the heart became very slow and the pressure fell. Gradually the pressure rose again until suddenly the block disappeared and the blood pressure was fully restored.

Bakhsh (1936) suggested that the cardiac slowing was central in origin, but since conessine depressed the action of acetylcholine in so many directions it seemed unlikely that this would be so. The effect of conessine on vagal stimulation was therefore tested. In the experiment illustrated in Fig. 11 the injection of 8 mg. conessine abolished the fall of blood pressure produced by vagal stimulation, and diminished the depressor effect of 20 μg. acetylcholine by cutting out the slowing of the heart which this dose produced. When the respiration was recorded by Gaddum's method (1941), 8 mg. conessine was observed to diminish both rate and depth (see Fig. 10).

It is worth while to record the great tolerance of rabbits to the intravenous infusion of *iso*conessine at a uniform rate. No less than 106 mg. was infused into one rabbit, the heart continuing to beat strongly; only when the rate of infusion exceeded 2.5 mg. per min. was the heart seriously affected. Solmann (1942) states that quinidine leaves the blood stream very rapidly, and it may be that *iso*conessine does so also.

Spasmolytic action.—The spasmolytic action of four local anaesthetics and of quinidine was compared with that of atropine by de Elío (1948). He found that quinidine was six times more powerful than procaine, and procaine was equal to cocaine. Conessine has been stated both to relax and to stimulate smooth muscle but no one has hitherto shown that it depresses the action of acetylcholine. Fig. 12 is a record of the contractions of an isolated piece of rabbit intestine, in which it will be seen that in the presence of concentrations of conessine from 10^{-5} to 2×10^{-6}, the stimulant action of acetylcholine is proportionally reduced. Both *neo*conessine and *iso*conessine were found to be slightly stronger than conessine, and about equal to one another. When a comparison was made between conessine and quinidine on a loop of guinea-pig ileum, it was found that conessine was slightly less potent than quinidine.

Antimalarial action.—In view of the close similarity of conessine and its isomers to quinine and quinidine, a comparison was made with quinine in chickens infected with *Plasmodium gallinaceum*. This test was done under the supervision of Mr. L. G. Goodwin in the Wellcome Research Institute. Chicks, 6 days old, were inoculated intravenously with 0.2 ml. blood

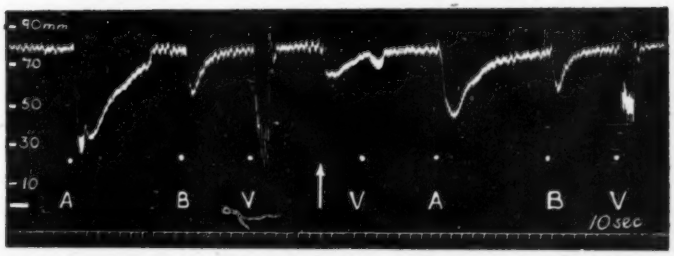


FIG. 11.—Rabbit, urethane. Record of blood pressure. At A 20 μg. ACh were injected intravenously, at B 1 μg. ACh was injected and at V the peripheral end of the right vagus was stimulated for 15 sec. At the arrow, 8 mg. conessine was injected and this abolished the effect of the vagus on the heart. The heart was no longer affected by 20 μg. ACh, though the injection still produced a fall of pressure. After 5 min. the heart was again sensitive to vagal stimulation but the effect was not as great as previously.

diluted to contain 10^8 parasitized red cells. The alkaloids were given by mouth, once on the day of inoculation, and twice on each of the three following days; 17 hours after the last dose, a blood smear was prepared from each bird in which 200 red cells were examined to determine the percentage which contained parasites. The results, recorded in Table II, indicate that, in the doses used, no antimalarial effect was exerted by conessine or its isomers.

TABLE II

ANTIMALARIAL ACTION IN CHICKS INFECTED WITH *P. Gallinaceum*

Substance	Dose mg./kg.	No. of birds	Mean percentage of cells with parasites
(Controls)	—	7	42
Conessine	40	4	32
,, ..	20	4	51
*Iso*conessine	80	4	46
,,	40	8	37
,,	20	8	42
*Neo*conessine	40	3	38
,,	20	3	21
Quinine ..	40	8	0.5
,, ..	20	8	11.4

Toxicity.—Conessine and its isomers were compared with one another by intravenous injection into mice in order to determine the mean lethal dose. Care was taken to inject at a uniform rate. For conessine 39 mice were used, for *iso*conessine 30 mice, and for *neo*conessine 50 mice. The lethal dose killing 50 per cent of mice was for conessine 28.7 mg. per kg., for *iso*conessine 33.2 mg. per kg., and for *neo*conessine 13.1 mg. per kg. Thus whereas conessine and *iso*conessine are very similar in toxicity, *neo*conessine is more than twice as toxic. It may be emphasized that while the actual figure for the toxicity of any one compound may vary greatly in different laboratories, the relative toxicities of the three compounds should remain constant.

DISCUSSION

Recent papers from this laboratory (Dawes, 1946; Dews and Graham, 1946; de Elío, 1948; Dutta, 1948) have brought out the similarity between various substances not previously considered to be related. Dawes's work showed that substances with a quinidine-like action on the heart included various local anaesthetics, spasmolytics and analgesics, and that quinidine and procaine reduced the action of acetylcholine in cardiac, skeletal and unstriped muscle. De Elío added to the evidence by testing four local anaesthetics, as well as atropine, pethidine and quinidine, on the frog rectus, the rabbit auricles and the rabbit intestine. In general all seven substances reduced the action of acetylcholine in these tissues and a close parallelism between local anaesthetic action and effect on the frog rectus was found to exist. Dutta tested pethidine, quinidine, atropine and benadryl to see whether they possessed the property, which procaine has long been known to possess, of lowering body temperature. He found that all these substances resembled procaine in this respect.

When conessine and its isomers were to be examined, their known local anaesthetic activity suggested that they also should be examined from the point of view described, and that since quinidine was also a plant alkaloid with local anaesthetic properties, the relation of their action to

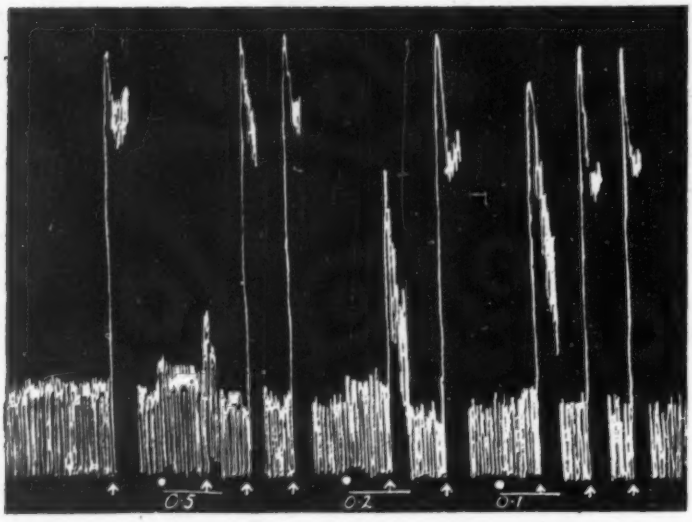


FIG. 12.—Isolated rabbit intestine, Ringer-Locke 36° C. The contractions at the small arrows were produced by the addition of 8 μg. ACh to the bath. Contractions produced in the presence of 0.5 mg., 0.2 mg. and 0.1 mg. of conessine (added at the white spot) were reduced in proportion to the amount of conessine. The fluid in the bath was changed at the end of the horizontal lines.

that of quinidine should be considered with special care. The outcome has been to demonstrate that in many respects conessine and its isomers are very similar to quinine and quinidine. So close indeed did the resemblance seem at one point that tests for anti-malarial activity were carried out. However, in doses in which quinine was active, conessine and its isomers were inactive. They have not yet been tested in higher doses.

Conessine resembles quinidine in diminishing the action of acetylcholine on the isolated intestine, the rabbit auricle, the frog rectus and on denervated mammalian muscle. The contractions of the rat diaphragm produced by nerve stimulation are occasionally augmented by *iso*conessine as they usually are by quinidine; the usual effect of conessine is depression and this is sometimes seen with quinidine. Conessine, like quinidine, lengthens the refractory period of cardiac tissue, and the effect of vagal stimulation on the heart in the anaesthetized rabbit is temporarily abolished by conessine as by quinidine (Starr, 1936) and by quinine in the dog (Babkin and Ritchie, 1945).

Of considerable interest is the parallelism between local anaesthetic action and inhibition of the action of acetylcholine on the frog rectus. This was first observed by de Elío, and the observations on conessine and its isomers make the parallelism still more complete. This is a further addition to the evidence that the action of acetylcholine is concerned with the sensation of pain. Harvey, Lilienthal and Talbot (1941) found that when acetylcholine was injected intra-arterially in men it caused very severe pain. Gray (1947) has detached a portion of the skin of an anaesthetized cat so as to leave only the artery, vein and nerve in connexion with the body. When he injected acetylcholine into the artery, he found that an action potential was set up in the nerve, and that this action potential was similar to that produced by mechanical pressure on the detached portion of skin. Thus a local anaesthetic may be a substance which reduces the action of acetylcholine at sensory nerve endings, and this would explain why local anaesthetic potency is related to the reduction of the action of acetylcholine on the frog rectus.

Hitherto conessine has been thought to be an alkaloid with its own peculiarities unlike those of any other plant alkaloid. It is now evident that this is not so, and that the action of conessine, which comes from the bark of Holarrhena, is very similar to that of the alkaloids quinidine and quinine which come from the bark of Cinchona. The actions of conessine also resemble those of papaverine, cocaine and atropine, three other plant alkaloids, all of which are local anaesthetics, spasmolytics and have a quinidine-like action on the heart. It is an obvious task to look at still more plant alkaloids in order to see how many others can be brought into this class, and to examine them for points of chemical and physical similarity.

SUMMARY

1. Conessine and its isomers, *iso*conessine and *neo*conessine, have been shown to possess properties very similar to those possessed by quinine and quinidine. Conessine, however, has no antimalarial action in chickens when tested in the same dose as quinine.

2. The local anaesthetic potency of conessine and its isomers is great; when tested by intracutaneous injection into guinea-pigs, conessine is about twice as active as cocaine, *iso*conessine is 50 per cent stronger than cocaine, and *neo*conessine is about equal to cocaine. The relative local anaesthetic potencies of conessine, its isomers, cocaine, quinidine and procaine are very similar to their relative activities in depressing the action of acetylcholine on the frog rectus muscle.

3. Conessine and its isomers have a quinidine-like action on the heart, and depress the action of acetylcholine on skeletal, cardiac and smooth muscle including that of the blood vessels, acting on all tissues very much like quinidine.

This work has been done while in receipt of a grant from the Therapeutic Research Corporation, to whom I wish to express my thanks. I am also indebted to Dr. S. Siddiqui for a supply of conessine, *iso*conessine, and *neo*conessine. The work has been carried out under the direction of Prof. J. H. Burn, and this paper has been written with his help.

References

Babkin, B. P., and Ritchie, T. W. (1945). *Rev. Canad. Biol.*, **4**, 346.
Bakhsh, I. (1936). *J. Pharmacol.*, **58**, 373.
Bülbring, E. (1946). *Brit. J. Pharmacol.*, **1**, 38.
Bülbring, E., and Wajda, I. (1945). *J. Pharmacol.*, **85**, 78.
Burn, J. H. (1914). *J. Pharmacol.*, **6**, 305.
Chopra, R. N. (1933). *Indigenous Drugs of India*. Calcutta: The Art Press.
Chopra, R. N., Gupta, J. C., David, J. G., and Ghosh, S. (1927). *Ind. Med. Gaz.*, **62**, 132.
Dawes, G. S. (1946). *Brit. J. Pharmacol.*, **1**, 90.
Dews, P. B., and Graham, J. D. P. (1946). *Brit. J. Pharmacol.*, **1**, 278.
Dutta, N. K. (1948). *Brit. J. Pharmacol.*, **3**, 246.
Elfo, F. J. de (1948). *Brit. J. Pharmacol.*, **3**, 108.
Gaddum, J. H., and Kwiatkowski, H. (1938). *J. Physiol.*, **94**, 87.
Gaddum, J. H. (1941). *J. Physiol.*, **99**, 257.
Gray, J. A. B. (1947). *J. Physiol.*, **106**, P11.
Harvey, A. M. (1939). *J. Physiol.*, **95**, 45.
Harvey, A. M., Lilienthal, J. L., Jr., and Talbot, S. A. (1941). *Bull. Johns Hopkins Hosp.*, **69**, 529.
Keidel, A. (1878). *Uber die Physiol. Wirkung d. Conessin*, Inaugural Dissertation, Göttingen.
Sollman, T. (1942). *A Manual of Pharmacology. Philadelphia*: W. B. Saunders.
Starr, I. (1936). *J. Pharmacol.*, **56**, 77.
Stephenson, R. P. (1948). *J. Physiol.*, **107**, 162.
Trevan, J. W., and Boock, E. (1927). *Brit. J. exp. Path.*, **8**, 307.

Brit. J. Pharmacol. (1948), **3**, 246.

THE ACTION OF SUBSTANCES WHICH ANTAGONIZE ACETYLCHOLINE ON THE BODY TEMPERATURE OF MICE, BEFORE AND AFTER ADRENALECTOMY

BY

N. K. DUTTA

From the Department of Pharmacology, Oxford

(Received December 5, 1947)

In 1931 Glaubach and Pick described the fall of temperature produced in guinea-pigs by the injection of procaine. Recently Peczenik (1947) has extended this observation to castrated mice, and has shown in addition that the fall is greater after adrenalectomy. By administration of desoxycorticosterone acetate, progesterone, and other steroids before adrenalectomy, the mice were protected to a varying extent, so that the fall of temperature due to procaine was smaller.

No light has hitherto been shed on the reason for this action of procaine. Peczenik says that the fall is probably the result of a shock to the autonomic nervous system. Recently, however, attention has been drawn to other properties possessed by procaine in addition to its local anaesthetic action: Dawes's work (1946) on quinidine substitutes led him to point out that quinine, quinidine, and procaine reduce the action of acetylcholine on the intestine, on the heart, and, as was shown by Harvey (1939a and b), on skeletal muscle. Dawes showed that procaine, atropine, and pethidine act like quinidine on the electrically driven auricle, and having pointed out the local anaesthetic action of atropine, which has long been known, he demonstrated that pethidine possesses a local anaesthetic action too. Thus atropine, pethidine, procaine, and quinidine are four substances having several properties in common, and de Elío (1948) has determined their relative potency in reducing the action of acetylcholine on the frog rectus, the rabbit intestine, and on the isolated rabbit auricle. The question then arose whether atropine, pethidine, and quinidine would also share with procaine the ability to cause a fall of body temperature in mice. The experiments here described supply the answer. The antihistamine substance benadryl was also included in the investigation because of the observations of Dews and Graham (1946); they tested the antihistamine substance neoantergan, which is known to have some atropine-like action, and found it to possess a quinidine-like action on the auricle, and a local anaesthetic action. I have found that neoantergan also depresses the action of acetylcholine on the frog rectus and the rabbit auricle, though the doses required are rather large. Benadryl also has been shown to have a local anaesthetic action (Leavitt and Code, 1947) and to reduce the action of acetylcholine on the intestine (Loew, MacMillan, and Kaiser, 1946).

METHODS

Since Peczenik has described the conditions for observing the fall of temperature produced by procaine in mice, his procedure was followed in several respects. Male mice weighing not less than 26 g were used, and all were castrated. Peczenik states that non-castrated mice "did not behave uniformly." They had access to food and water except during the observation of body temperature. They were fed on a diet of constant composition consisting of ground grain; its percentage composition was: wheat 19, bran 19, oats 14, maize 9, barley 9, fish-meal 5, bone-meal 9, skimmed milk 14, yeast 1, salt 0.5, and cod-liver oil 0.5 per cent. The mice were kept at about 25° C. and experiments were carried out at 25° ± 1° C. in a small room with a thermostat.

The rectal temperature of each mouse was recorded by inserting a thermocouple into the rectum for 2.5 cm.; this thermocouple was connected to a moving coil galvanometer, and the other thermocouple was placed in melted sodium sulphate crystals in a thermos flask. The thermocouple in the rectum of the mouse was kept in position by adhesive tape fixed to the tail, and the tail was fixed along its length to a copper wire which continued forwards over the back of the mouse as far as the forelegs, where it turned around the body of the mouse in a ring behind

them ; the mouse was thus held comfortably throughout the period of observation and passed faeces as usual.

The temperatures of 8 mice were followed in each experiment during the same period of time ; 4 mice were used as controls and were injected with saline, while 4 were injected with the substance being tested. The injections of atropine, pethidine, and procaine were subcutaneous, while those of benadryl and quinidine were intraperitoneal. Before each experiment all mice were kept in the room until their temperatures remained steady ; this usually required 30–45 min.

RESULTS

Pethidine (demerol).—In order to illustrate the performance of the tests, the results with one dose of pethidine will be given in detail so that the remaining results can be given in summarized form.

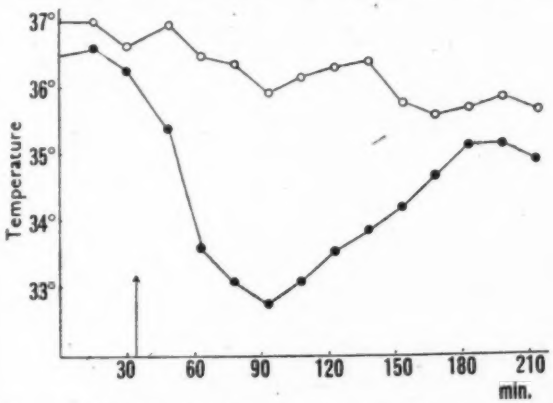


FIG. 1.—Abscissae: time in min.; ordinates: body temperature in °C. White circles, mean body temperature of 4 control mice injected at arrow with saline. Black circles, mean body temperature of 4 mice injected at arrow with pethidine hydrochloride, 30 mg./kg.

Observations were made on three groups of 8 castrated mice, half of which received 30 mg./kg. pethidine hydrochloride, and half received the same volume of saline. The results in one of these groups are given in Fig. 1. The body temperature of the control mice fell during the 3 hours after the injection from a mean value of 36.6° C. to a mean value of 35.6° C. The temperature of the mice injected with pethidine reached a minimum of 32.7° C. one hour after injection and slowly recovered to 35.2° during the next 1½ hours. The results in the other two groups were similar to those shown in Fig. 1 ; they are given in Table I. It will be noted that the fall of temperature in the control mice was greater in both groups than that in Fig. 1. In Group 2 the mean temperature fell from 35.5 to 33.8°, while in Group 3 it fell from 34.6 to 32.9° in the course of the experiment. In

TABLE I

TEMPERATURES OF MICE RECEIVING 30 MG./KG. PETHIDINE HYDROCHLORIDE

Time min.	Group 2			Group 3		
	Control	Injected	Difference	Control	Injected	Difference
0	35.5	35.5	0	34.6	35.8	−1.2
15	35.9	35.1	0.8	34.6	35.0	−0.4
30	35.1	33.7	1.4	34.3	33.8	0.5
45	34.7	32.6	2.1	34.2	33.2	1.0
60	34.6	32.3	2.3	34.3	32.9	1.4
75	34.5	32.6	1.9	34.1	32.8	1.3
90	34.4	33.1	1.3	33.7	32.8	0.9
105	34.4	33.1	1.3	33.7	33.0	0.7
120	34.2	33.5	0.7	33.3	32.5	0.8
135	34.2	33.6	0.6	33.0	32.5	0.5
150	34.2	34.5	−0.3	33.1	32.6	0.5
165	33.8	34.1	−0.3	32.9	32.6	0.3

Table I the differences between the control mice and those injected with pethidine are also shown. The mean differences for the three groups were calculated and the resulting figures are plotted as ordinates against time as abscissa in Fig. 2. In this and succeeding Figures, the mean difference of temperature between the two groups of mice before

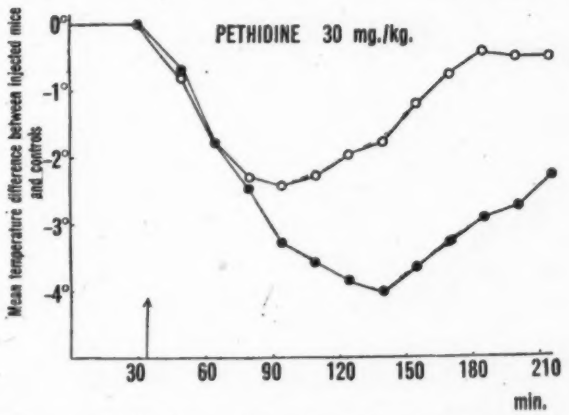


FIG. 2.—Ordinates: difference between mean body temperature of mice injected with 30 mg./kg. pethidine hydrochloride and of control mice, taking difference before injection as zero and correcting throughout for this difference. White circles show observations on 12 injected and 12 control mice before adrenalectomy. Black circles show observations on 9 injected and 8 control mice after adrenalectomy.

injection is shown as 0° ; this was often not actually so (see Fig. 1), but the difference of temperature between the two groups throughout the period of observation was corrected by the difference which existed before injection.

The mice used in obtaining these results were kept for one week after the observations were

made ; adrenalectomy was then carried out under ether. Two days after the operation the effect of the same dose of pethidine was redetermined. Of the 24 mice tested before the operation, 7 died during the next two days, so that only 17 were available ; of these 8 were injected with saline and 9 were injected with pethidine. The differences of temperature between the mice receiving pethidine and the control mice are recorded in the lower curve of Fig. 2. Fig. 2 shows that after adrenalectomy the fall of temperature caused by pethidine was much greater than before.

Observations were also made with a higher dose of pethidine, namely 100 mg./kg. In the mice before adrenalectomy this dose produced a rise of body temperature, the mean maximum rise in 12

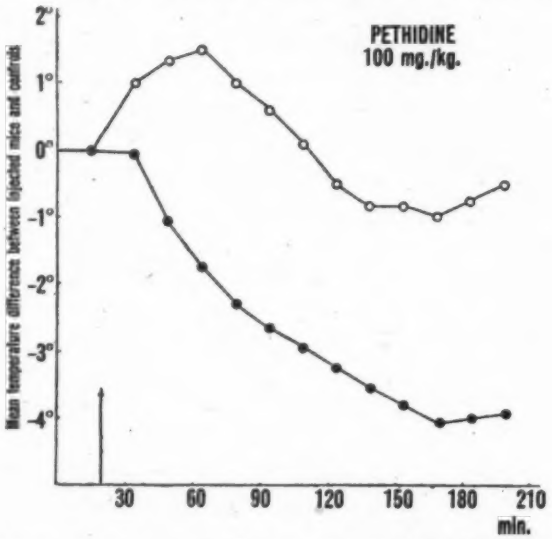


FIG. 3.—Similar to Fig. 2. Dose of pethidine 100 mg./kg. Note the rise of temperature before adrenalectomy when 12 mice were compared with 12 controls. Black circles show results after adrenalectomy; (10 mice injected and 9 controls).

animals being to a point 1.5° above the mean temperature of the 12 control mice. The rise, however, subsided after 1.5 hours and was followed by a fall. The change is shown in Fig. 3. This high dose of pethidine not only produced a rise of temperature but also caused increased muscular movements in all the mice.

The rise disappeared after adrenalectomy: 10 mice were injected with pethidine and 9 were injected with saline. The pethidine caused a large fall of temperature relative to the temperature of the control mice ; this is shown in the lower curve of Fig. 3.

Atropine.—Similar experiments were then made with atropine, using first of all 150 mg./kg. of atropine sulphate. This dose was too high ; in two of the three groups used, the dose was fatal to the mice after adrenalectomy. In one of the groups, however, the tests were carried out successfully, and, though the maximum fall of temperature before adrenalectomy was only 1.3° more than in the controls, after adrenalectomy the maximum fall was 3.2° more than in the controls. A further

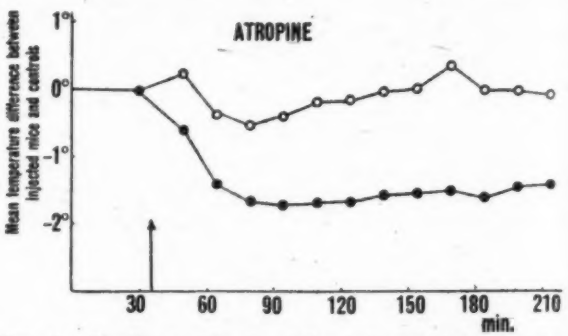


FIG. 4.—Similar to Fig. 2. Mice injected with atropine sulphate 30 mg./kg. White circles, 12 injected and 12 control mice before adrenalectomy. Black circles, 10 injected and 10 control mice after adrenalectomy.

test was then made using 30 mg./kg. atropine sulphate, and the results which were obtained in 24 mice before adrenalectomy and in 20 of these after adrenalectomy are given in Fig. 4. Before the operation the fall of temperature was slight, the maximum being 0.5° more than the controls. This is an effect too small to be discovered except by careful investigation. After adrenalectomy it was increased to about 1.5°, and this was maintained.

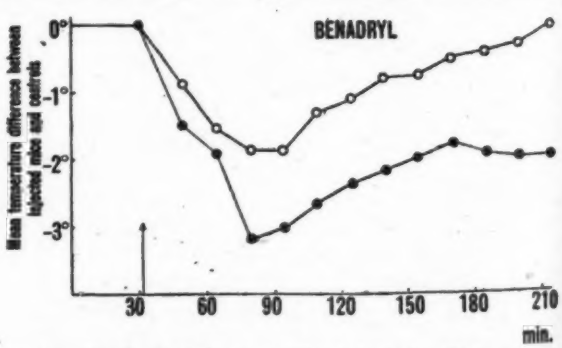


FIG. 5.—Similar to Fig. 2. Mice injected with benadryl 20 mg./kg. White circles, 8 injected and 8 control mice before adrenalectomy. Black circles, 7 injected and 7 control mice after adrenalectomy.

Benadryl.—Results with the hydrochloride of β-dimethylaminoethylbenzhydrylether, which is called benadryl, are shown in Fig. 5 ; they were obtained with a dose of 20 mg./kg. Before adren-

alectomy 16 mice were used, and after adrenalectomy 14 of the same mice were used. The results were similar to those obtained with the lower dose of pethidine, though the fall of temperature was less.

Procaine.—Peczenik injected his mice with procaine hydrochloride in a dose of 100 mg./kg. When this amount was used, the fall of temperature was very small, and was not much greater after adrenalectomy. The maximum fall in 8 mice compared with 8 controls was 0.5° before and

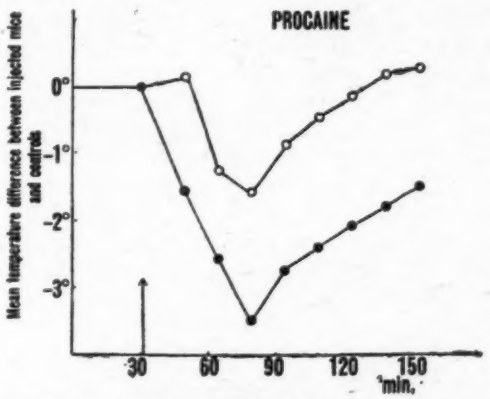


FIG. 6.—Similar to Fig. 2. Mice injected with procaine hydrochloride 200 mg./kg. White circles, 8 injected and 8 control mice before adrenalectomy. Black circles, 8 injected and 6 control mice after adrenalectomy.

1.5° after the operation. Further observations were then made with 200 mg./kg., and the results in 8 mice, compared with 8 controls, are shown in Fig. 6. The difference in sensitiveness between mice in this laboratory and those in Peczenik's laboratory is not surprising, for similar differences are often seen.

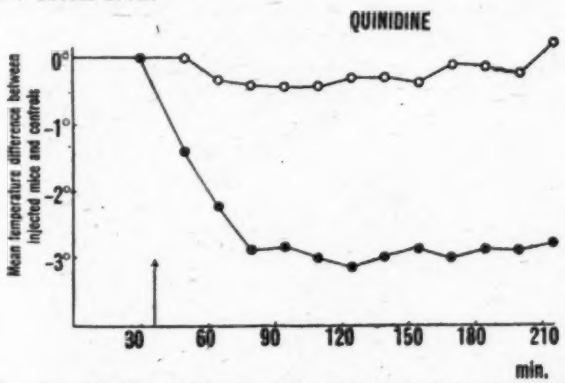


FIG. 7.—Similar to Fig. 2. Mice injected with quinidine hydrochloride 30 mg./kg. White circles, 12 injected and 11 control mice before adrenalectomy. Black circles, 9 injected and 7 control mice after adrenalectomy.

Quinidine.—The final observations were made when quinidine hydrochloride was given in a dose of 30 mg./kg. As after atropine, there was a very small fall of temperature before adrenalectomy, not exceeding 0.5°. After the operation there was, however, a fall of 3°, which was maintained throughout the period of observation. The results are shown in Fig. 7.

DISCUSSION

So far as the action of procaine is concerned, the results described confirm the finding of Peczenik that procaine causes a fall of body temperature in mice and that this fall is greater after adrenalectomy. However, the " collapse " of temperature which Peczenik observed after adrenalectomy was not seen ; the temperature fell, but so did that of many control mice, and the effect of procaine was not great. Peczenik found the mean fall in 20 mice after adrenalectomy to be 4.9° when procaine was given in the dose 100 mg./kg. ; he does not record the temperature changes in control mice kept alongside. In the work here described, the maximum difference between injected mice and control mice was found to be only 1.5° after 100 mg./kg., and only 3.5° after twice this amount. The difficulty which a mouse has in maintaining a constant temperature has been discussed by Fuhrman (1946) ; on account of its large surface per unit weight, the body temperature falls more easily than in larger animals, and control observations are therefore essential.

The main result of this research is to demonstrate that the property which procaine possesses of reducing temperature is shared by a number of other substances—atropine, quinidine, pethidine, and benadryl. The experiments were undertaken in order to see if this would be so, and the hypothesis concerning their action has thus gained additional support. It was pointed out that all these drugs reduce or abolish the action of acetylcholine on the frog rectus, on the spontaneous contractions of the rabbit auricle, and on the rabbit intestine. Further, atropine, procaine, and pethidine act like quinidine on the refractory period of the electrically driven auricles of the rabbit (Dawes, 1946). I myself have observed that benadryl reduces the effect of acetylcholine on the frog rectus and the rabbit auricle, and I have found that it lengthens the refractory period. Since de Elío (1947) has shown that acetylcholine shortens the refractory period, the quinidine-like action of this group, if not one which can be described as a depression of the action of acetycholine, is at least an action in the opposite direction to that of

acetylcholine. Thus there is some ground for the view that the common property of reducing body temperature may be connected with the power to reduce or antagonize the action of acetylcholine.

It is at least clear that the common property is not due to the possession of a local anaesthetic action, though all of these substances have one. Thus it was found by Glaubach and Pick (1931) that while cocaine caused a varying change of body temperature—namely, a rise in rabbits and a fall in guinea-pigs—nupercaine, one of the most potent local anaesthetics, had almost no effect on the temperature of either species. This is of interest because de Elío found that nupercaine did not reduce the action of acetylcholine on the rabbit auricles when spontaneously contracting, no matter in what dose nupercaine was applied. Thus nupercaine provides an example of a local anaesthetic which differs from procaine in reducing neither body temperature nor the action of acetylcholine in the cardiac tissue of the rabbit.

The conclusion that substances which reduce body temperature are also substances which reduce the action of acetylcholine must not be taken to imply that acetylcholine plays a role in the central nervous system to maintain body temperature. Feitelberg, Pick, and von Warsberg (1939) tested the action of acetylcholine on the temperature of the grey matter of the cortex of cats and found that sometimes it caused a fall and sometimes a rise. The fall which procaine, pethidine, etc., cause may be due to their inhibition of the action of acetylcholine in the skeletal muscles, on the activity of which the normal formation of heat depends. This suggestion is borne out by the fact that the large dose of pethidine which caused an initial rise of temperature also caused increased motor activity. The lower dose of pethidine, and also the doses of the other substances used, were too small to cause any visible effect in the mice.

The effect of adrenalectomy in augmenting the fall of temperature produced by these substances shows clearly that the adrenal glands are concerned in temperature control. Whether it is the cortex or the medulla which is the more important is not known for certain, though the power of cortical extracts to prevent the fall of temperature in adrenalectomized rats when exposed to low temperature indicates the greater importance of the cortex. Moreover, Peczenik claims to have shown that the administration of desoxycorticosterone acetate diminished the effect of removing the glands on the procaine fall of temperature.

A word is required in conclusion on the use of castrated mice. Observations on 16 normal male mice, 8 of which were injected with 30 mg./kg. pethidine and 8 of which were controls, gave a result almost the same as that obtained with castrated mice.

Summary

1. The finding that procaine causes a fall of body temperature in mice, and that this fall is greater after adrenalectomy, has been confirmed.

2. It has been shown that this property of procaine is shared by the analgesic pethidine (demerol), by quinidine, by atropine, and by the antihistamine substance benadryl.

3. The fall of temperature produced by each of these substances is augmented by adrenalectomy.

4. All these substances reduce or abolish the action of acetylcholine on the frog rectus, the rabbit auricle, and the rabbit intestine. It is suggested that the fall of temperature may be due to lessened activity and lessened heat production in the skeletal muscles of the mice.

5. A large dose of pethidine causes a rise of temperature accompanied by increased motor activity.

I am deeply indebted to Prof. J. H. Burn for suggesting this problem and for the guidance and encouragement he has given in all the stages of this work.

This work was carried out during the tenure of a grant from the Health Department, Government of India.

References

Dawes, G. S. (1946). *Brit. J. Pharmacol.*, **1**, 90.
Dews, P. B., and Graham, J. D. P. (1946). *Brit. J. Pharmacol.*, **1**, 278.
Elío, F. J., de. (1947). *Brit. J. Pharmacol.*, **2**, 131.
Elío, F. J., de. (1948). *Brit. J. Pharmacol.*, **3**, 108.
Feitelberg, S., Pick, E. P., and von Warsberg, A. (1939). *Arch. intern. Pharmacodyn.*, **61**, 447.
Fuhrman, G. J. (1946). *Physiol. Rev.*, **26**, 247.
Glaubach, S., and Pick, E. P. (1931). *Arch. exp. Path. Pharmak.*, **162**, 551.
Harvey, A. M. (1939a). *J. Physiol.*, **95**, 45.
Harvey, A. M. (1939b). *Bull. Johns Hopk. Univ.*, **65**, 223.
Leavitt, M. D., and Code, C. F. (1947). *J. Lab. clin. Med.*, **32**, 334.
Loew, E. R., MacMillan, R., and Kaiser, M. E. (1946). *J. Pharmacol.*, **86**, 229.
Peczenik, O. (1947). *Proc. roy. Soc.*, **134B**, 218.

Brit. J. Pharmacol. (1948), **3**, 251.

THE INFLUENCE OF POTASSIUM AND CALCIUM IONS ON THE ACTION OF PROCAINE

BY

H. J. BEIN

From the Department of Pharmacology, Oxford

(Received February 24, 1948)

It has long been established that the optimum functioning of cells is dependent on optimum amounts of different electrolytes in the fluid surrounding them. In particular it is known that function is still maintained if an excess of one electrolyte is compensated by a corresponding increase of another, the best-known pair being the potassium and calcium ions, chiefly studied in the heart by Ringer.

It is important, however, to realize that pharmacological investigations which consider the action of drugs in relation to this variable content of electrolytes are very different from observations of body functions only. Electrolytes may prevent or assist the action of a drug on a tissue, and it is not only the affinity of an electrolyte to a cell which is important, but also its affinity to the drug. In the complicated system composed of the cell, a variable content of electrolytes and the drug, with all their mutual relations, the response of the tissue will not necessarily follow the same laws as it would in the absence of the drug.

Two recent papers show that the absolute concentration of potassium is of great importance for some drug actions, more so than its relative concentration to calcium, i.e., the K/Ca ratio. Baker (1947) tested the interrelation between Ca, K and ouabain on the isolated heart and in the whole animal; Goffart and Brown (1947) investigated the correlation of K and adrenaline on the isolated striated muscle. In order to see whether the effects described are peculiar to these two drugs or have a more general significance I have tested the effect of changing the K and Ca concentrations on another drug action, namely that of procaine on the refractory period of the isolated rabbit auricles.

METHOD

The method of measuring the activity of pharmacological agents on the refractory period has been described in detail by Dawes (1946a). The auricles, carefully dissected from the heart of a rabbit, are suspended in a bath containing 100 c.c. of oxygenated Ringer-Locke at 29° C. The composition of the Ringer-Locke is as follows: NaCl 9.0 g.; KCl 0.42 g.; $CaCl_2$ 0.24 g.; $NaHCO_3$ 0.5 g.; dextrose 2.0 g.; distilled water 1,000 c.c.

The contractions of the auricles are recorded on a smoked drum. The auricles contract rhythmically by themselves and they can also be driven by break-shocks from an induction coil at any desired speed. The maximal rate is observed at which the auricles will follow electrical stimulation. The reduction or the increase in maximum rate caused by a drug is calculated and expressed as a percentage of the maximum rate before the drug was applied. This percentage is, within wide limits, independent of the initial maximum rate, as Dawes observed.

RESULTS

Procaine

Procaine reduces the maximum rate, i.e., it prolongs the refractory period of the isolated rabbit auricles (for references see Dawes, 1946a and b).

The activity of procaine was tested on 9 preparations. The effect was measured after the procaine had been allowed to act for a fixed time. The average results of all observations are shown in Table I.

TABLE I

Concentration of procaine acting for 10 minutes	3×10^{-6}	10^{-5}	2×10^{-5}
Percentage reduction of the maximal rate	13·8 ± 0·4	23·9 ± 0.98	34·4 ± 0.88

The effects of procaine were clear, easy to estimate, and the muscle recovered in 25–30 minutes. This relatively rapid recovery was one of the reasons why procaine was chosen rather than any of the other substances with "quinidine-like properties" tested by Dawes (1946a and b).

The spontaneous rhythm was slightly increased, or remained unaltered, by a low concentration of procaine (3×10^{-6}), while it was decreased by a higher concentration (2×10^{-5}); this effect was more pronounced if the original pulse-rate was high.

Potassium and calcium ions

Potassium is reported to be without effect on the refractory period of the frog ventricle (Kanda, 1939), although many authors have found that it lengthens the refractory phase (Boehm, 1914; Cicardo and Marenzi, 1938; Lueken and Schuetz, 1939). Calcium has been found to be without any clear effect (Cicardo and Marenzi, 1938).

In my experiments an increase in potassium lengthened the refractory period; for example, the maximum rate was reduced by 23 per cent and the spontaneous rate by 25 per cent, if the potassium concentration of the Locke solution was raised by 50 per cent.

If the auricle was left beating in potassium-free Ringer solution for about 2 hours this had no effect on the refractory period; there was, however, an effect on the pacemaker, since the spontaneous rhythm increased by 40 per cent.

When the amount of $CaCl_2$ was doubled or tripled, the maximal rate increased by no more than 3–7 per cent, and even very high concentrations (8 times normal) either did not affect the refractory period or lengthened it by about 5 per cent only, at a time when the spontaneous rate was increased by 47 per cent.

Reduction of the calcium concentration by 50 per cent prolonged the refractory period; the maximum rate at which the auricle responded to electrical stimuli was reduced by 15 per cent. This decrease resembled the effect on the refractory period produced by increasing the potassium content, but while the pacemaker was depressed by excess K, it was not affected by lowering the calcium concentration to 50 per cent.

Effect of K and Ca on the action of procaine

When the influence of changes in K or Ca content on the action of procaine was tested, the change of electrolyte concentration was first allowed to establish its own intrinsic effect for a period of 1 to 3 hours before the procaine was added; this was done in order to ensure that the result observed was not due to summation of the effects of the electrolyte and of procaine. Increasing the amount of potassium (Fig. 1A) or decreasing the amount of calcium (Fig. 1B) both potentiated the action of procaine. About the same proportional change was obtained by raising the K by 50 per cent or by lowering the Ca by 50 per cent.

A reduction of the potassium or an increase of the calcium content produced the same effect qualitatively but not quantitatively; in these conditions the action of procaine was depressed. However, in order to obtain the same degree of depression as that produced by reducing the K concentration by 50 per cent, it was necessary to increase the Ca concentration by 600 per cent (see Fig. 1C and D); for example, the effect of 2×10^{-5} procaine in Locke solution containing half the normal amount of K was depressed by 66 per cent, but a depression of 64 per cent was only produced by raising the Ca concentration to 7 times the normal amount. In another experiment even 8 times more Ca was required.

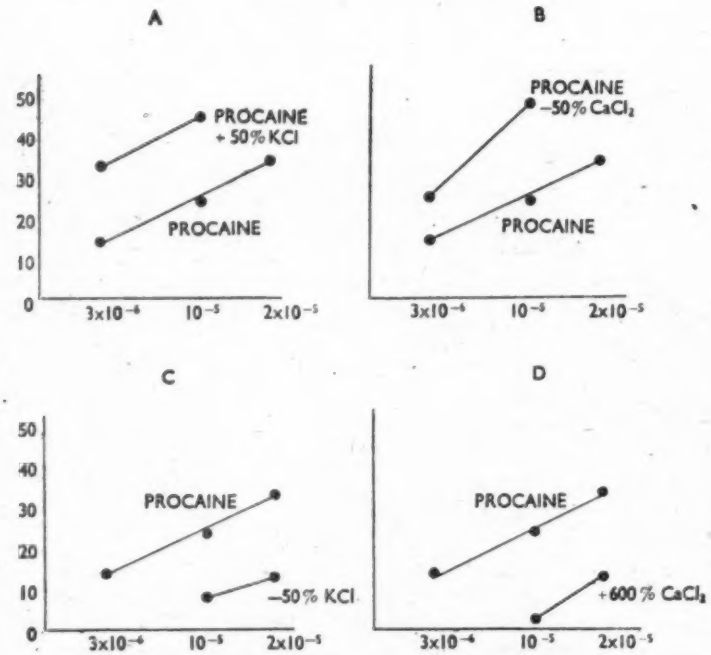


FIG. 1.—Effect of procaine on isolated rabbit auricles with different K and Ca concentrations. Ordinates: percentage reduction in the maximal rate at which the auricles follow electrical stimulation. Abscissae: concentration of procaine.

DISCUSSION

The experiments described show that the influence of potassium and calcium on the refractory period

of the isolated rabbit auricles is antagonistic. Although an excess of calcium or a reduction of potassium is without intrinsic effect over a period of 2–3 hours, this change of electrolytes nevertheless influences the action of procaine, demonstrating that an optimal amount of all electrolytes is necessary for the usual response of the cell to drugs, even though an intrinsic effect of variation in the contents of electrolytes itself is not immediately visible. The quantitative aspect, however, shows the importance of the absolute concentration of potassium for drug action. Baker (1947) has shown it for the action of ouabain, and in this paper it has been demonstrated for procaine. Under the experimental conditions of this investigation the alteration of procaine activity by a reduction of potassium cannot be reproduced by a correspondingly increased amount of calcium; on the other hand the effect of an increased amount of potassium can be imitated by a corresponding loss of calcium, which shows that at least the normal amount of Ca should be present.

Summary

(1) The action of procaine on the refractory period of the isolated rabbit auricle was determined in the presence of variable amounts of potassium and calcium.

(2) Qualitatively, an excess of potassium and a reduction of calcium potentiate the activity of procaine; a reduction of potassium and an excess of calcium diminish the effect of procaine on the refractory period.

(3) Quantitatively, the determining factor is not the ratio K/Ca but the absolute amount of potassium present, although a certain amount of Ca is necessary.

I should like to express my sincere thanks to Prof. J. H. Burn for his hospitality and the help given while carrying out this work.

References

Baker, J. B. E. (1947). *Brit. J. Pharmacol.*, **2**, 259.
Boehm, R. (1914). *Arch. exp. Path. Pharmak.*, **75**, 230.
Cicardo, v. H., and Marenzi, A. D. (1938). *Rev. Soc. Argentine Biol.*, **14**, 59.
Dawes, G. S. (1946a). *Brit. J. Pharmacol.*, **1**, 90.
Dawes, G. S. (1946b). *Brit. med. J.*, **1**, 43.
Goffart, M., and Brown, G. L. (1947). *C.R. Soc. Biol., Paris*, **141**, 958.
Kanda, Z. (1939). *Arch. exp. Path. Pharmak.*, **190**, 417.
Lueken, B., and Schuetz, E. (1939). *Z. Biol.*, **99**, 186, 339.

Brit. J. Pharmacol. (1948), **3**, 254.

THE CHANGE IN PHARMACOLOGICAL ACTION PRODUCED BY THE INTRODUCTION OF A METHYL GROUP INTO PRISCOL

BY

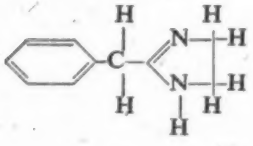
C. W. GOWDEY

From the Department of Pharmacology, Oxford

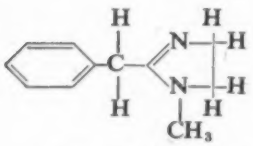
(Received February 25, 1948)

Within the last decade a new series of chemical compounds, the 2-substituted imidazolines, have claimed wide attention because of their vascular actions. Several of them, phedracin, priscol, privine and otrivin, have been used clinically in peripheral vascular disorders.

Hartmann and Isler (1939) made a preliminary pharmacological investigation of a large number of these imidazolines. They found that small changes in the imidazoline molecule sometimes caused great quantitative or even qualitative changes in the effect upon the blood pressure. Similar abrupt transitions have been described among sympathomimetic amines (Beyer, 1946). Since the substitution of an N-methyl group in the noradrenaline molecule causes significant differences in its properties (Barger and Dale, 1910) it was decided to investigate what changes the introduction of an N-methyl group would effect in priscol. The structural formulae of these compounds are as follows:



2-benzylimidazoline (priscol)



2-benzyl-1-methyl-imidazoline (N-methylpriscol)

2-Benzyl-1-methyl-imidazoline was one of the compounds investigated by Hartmann and Isler. They stated that it had approximately the same toxicity and effect on the rabbit's intestine as priscol, but caused a rise of blood pressure, whereas priscol caused a fall.

The general pharmacology of priscol and its N-methyl derivative is here described as part of an attempt to elucidate the mechanism of action of the imidazolines.

Experimental Results

Cardiovascular action of priscol.—The action of priscol on the blood pressure varies in different species. Meier and Müller (1939) observed a fall of blood pressure in rabbits; Hermann, Jourdan and Bonnet (1941) found that in dogs priscol usually caused a fall but occasionally a rise of blood pressure. In cats Chess and Yonkman (1945) observed either no effect or a fall with larger doses.

Since priscol is structurally related to histamine it seemed possible that the fall of blood pressure might be antagonized by neoantergan. However, in a cat under ether anaesthesia, a dose of neoantergan which virtually abolishes the depressor effect of histamine did not prevent the fall of blood pressure caused by a large dose of priscol. All the workers quoted above observed that priscol reduced or reversed the pressor action of adrenaline. An example of this in the spinal cat is shown in Fig. 1. After the injection of 10 mg. priscol, which was itself without action on the blood pressure or spleen volume, 10 μg. adrenaline caused a small fall of blood pressure accompanied by dilatation of the spleen; the pressor effect and the contraction of the spleen caused by 5 μg. adrenaline before the injection of priscol were absent. When a larger dose of adrenaline was injected, however, the original effect was seen

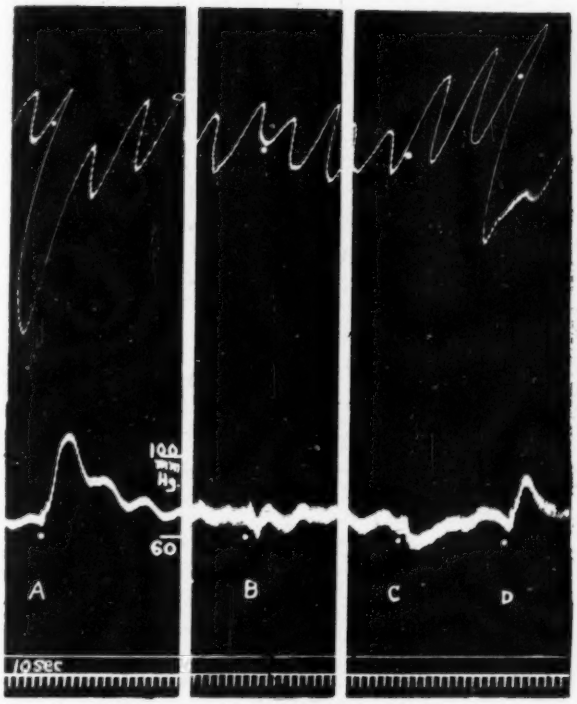


FIG. 1.—Upper record; spleen volume ; lower record: blood pressure of spinal cat. At A, 5 μg. adrenaline caused contraction of spleen and rise of pressure. At B, 10 mg. priscol. At C, 10 μg. adrenaline caused slight dilatation of spleen and fall of blood pressure. At D, 0.1 mg. adrenaline caused contraction of spleen and a small rise of pressure.

once more ; 0.1 mg. adrenaline caused a small rise of blood pressure and contraction of the spleen.

This observation suggested that the effect of priscol on the pressor response of adrenaline might be attributed to competition between priscol and adrenaline molecules for the same " receptor." The effect of different amounts of priscol on a range of doses of adrenaline was therefore determined in a series of spinal cats. The results are shown in Fig. 2 in which curve A shows the mean height of the pressor response to a given dose of adrenaline in the absence of priscol. Curve B shows the effect on the adrenaline response of administering priscol in a dose of 2.5 mg./kg., and curve C that of 5 mg./kg. It can be seen from Fig. 2 that the reversal of the pressor action of adrenaline by priscol is only possible when small doses of adrenaline are injected, and that the reversal is easily overcome by larger doses.

Priscol on blood vessels.—The action of priscol on the blood vessels was determined by perfusing the rabbit ear, using the method of Gaddum and Kwiatkowski (1938). In Fig. 3 the vasodilatation produced by 0.5 mg. priscol is shown. The action on the adrenaline response was also examined in this preparation and the record in Fig. 4 shows the reversal of the constrictor action when priscol was added to the Ringer's solution perfusing the ear: (*a*) shows two vasoconstrictor effects due to the injection of 0.1 μg. adrenaline ; at (*b*), in the presence of priscol, 0.8 μg. adrenaline caused

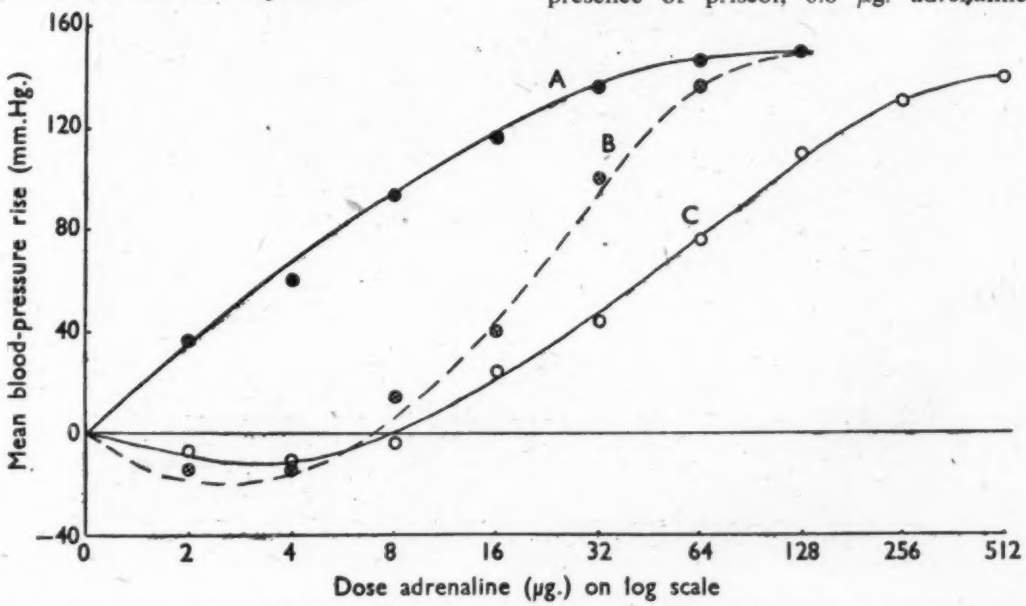


FIG. 2.—Priscol-adrenaline antagonism on blood pressure of spinal cat. Mean blood-pressure rise (ordinate mm. Hg.) plotted against dose (μg) adrenaline as abscissa on logarithmic scale. Curve A shows blood-pressure rise before priscol (mean of 3 exp.); curve B after 2.5 mg./kg. priscol (mean of 2 exp.); curve C after 5.0 mg./kg. priscol (mean of 3 exp.).

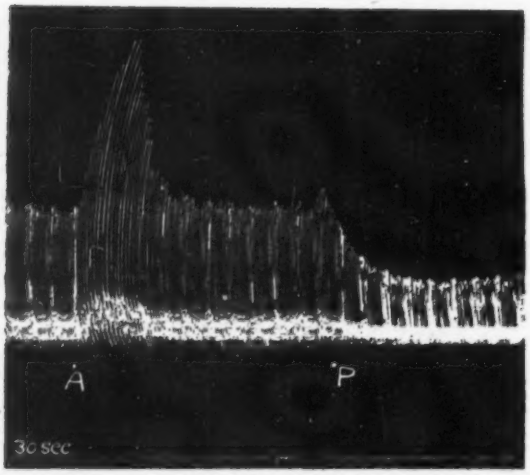


FIG. 3.—Record of outflow from vessels of rabbit ear obtained by Gaddum's drop timer. At A, 0.025 μg. adrenaline caused vasoconstriction; at P, 0.5 mg. priscol caused vasodilatation.

vasodilatation, and 1.6 μg. was without effect; at (*c*) 3.2 μg. adrenaline caused a slight vasoconstriction, and at (*d*) 4 μg. a constriction similar to the initial effects. Thus not only does priscol cause vasodilatation and reverse the vasoconstrictor action of adrenaline in isolated perfused vessels, but as in the spinal cat the relationship between priscol and adrenaline is quantitative and not qualitative. Histamine also causes vasoconstriction in the rabbit's ear; larger doses of priscol than those necessary to abolish the action of adrenaline abolished the vasoconstriction caused by histamine, but no reversal was seen.

Similar observations were made when the hind-leg of a dog was perfused with blood (containing heparin) by a Dale-Schuster (1928) pump. The injection of 10 mg. priscol caused vasoconstriction (in contrast to the vasodilatation seen in the rabbit's ear) and converted the previous constrictor action of 4 μg. adrenaline to a dilator action. When 20 mg. priscol was injected the dilator action of 4 μg. adrenaline became greater and resembled that of 20 μg. histamine. The effect of histamine itself was unchanged by priscol.

Priscol on the heart.—The action of priscol on the heart was examined by perfusing the isolated heart with Locke's solution by Langendorff's method. In two rabbit hearts, the injection of 10–20 μg. priscol decreased the amplitude and reduced the coronary flow. A similar action was observed in three experiments on cat hearts, but in other experiments on cat hearts an increase in the heart's action was observed especially with larger doses (1–3 mg.). An example of this is

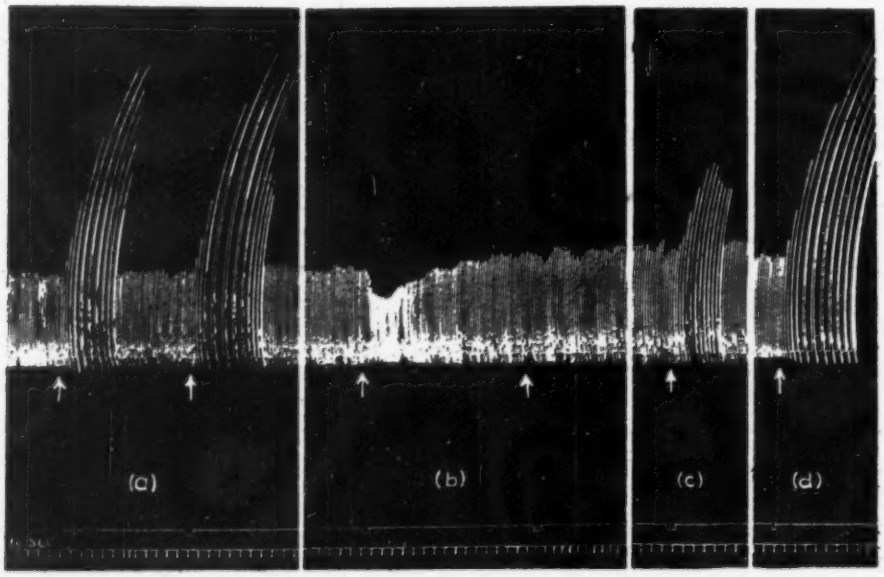


FIG. 4.—Record as in Fig. 3. In (*a*) two injections of 0.1 μg. adrenaline causing vasoconstriction. Between (*a*) and (*b*) perfusion with Ringer containing priscol (0.2 mg. per ml.) was begun. In (*b*) at first arrow 0.8 μg. adrenaline caused vasodilatation, and at the second, 1.6 μg. was without effect. In (*c*) 3.2 μg. caused vasoconstriction, and in (*d*) 4.0 μg. caused an effect similar to that in (*a*).

given in Fig. 5 which shows the effect of 2 mg.; this dose not only increased the amplitude but also both the rate (from 102–200 per min.) and the coronary flow (from 6.0–10.4 ml. per min.). Although the increase in amplitude did not last more than 8 min. the increase in rate and in coronary flow continued for 15 min.

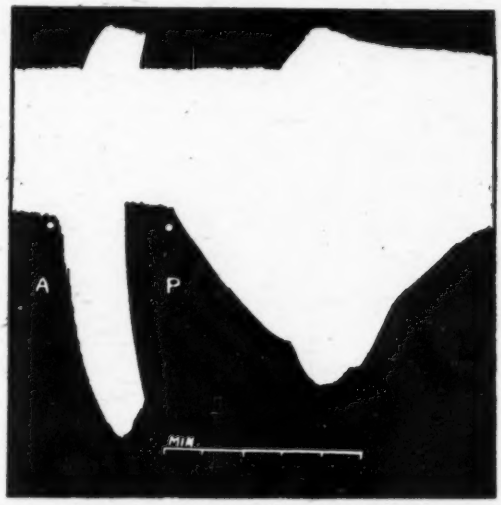


FIG. 5.—Record of contractions of isolated cat heart perfused by method of Langendorff. At A, 0.3 μg. adrenaline increased force of contraction; heart rate from 104/min. to 140/min., and coronary flow from 5.6 ml./min. to 7.8 ml./min. At P, 2 mg. priscol increased force of contraction; heart rate from 102/min. to 200/min., and coronary flow from 6.0 ml./min. to 10.4 ml./min. There is prolonged stimulation due to priscol: 8 min. after injection, heart rate still 173/min. and coronary flow 9.6 ml./min.

On isolated rabbit auricles at 29° C. priscol in concentrations of 2 μg.–4 μg. per ml. augmented the amplitude though it slowed the rate. Priscol lengthened the refractory period when examined by Dawes's method (1946a), though the concentrations required were large; 20 μg. per ml. increased it by 6 per cent., and 0.1 mg. per ml increased it by 22 per cent. Priscol did not affect the action of either adrenaline or acetylcholine on the spontaneous beats of the isolated auricles.

Cardiovascular action of N-methylpriscol.—The N-methyl derivative of priscol, unlike priscol, always caused a rise of blood pressure, as Hartmann and Isler (1939) had observed. An example is given in Fig. 6 in which the effect of injecting N-methylpriscol (20 mg.) is shown at A_1; the prolonged rise of blood pressure was accompanied by a great increase in heart rate. At B_1 10 μg. adrenaline was injected. A series of doses of nicotine was then given, until no further pressor action was observed; a total of 31.5 mg. nicotine acid tartrate was required. The injection of 10 μg. adrenaline at B_2 then caused about the same rise of blood pressure as at B_1, but the injection of 20 mg. N-methylpriscol at A_2 was almost without effect. This suggested that the pressor action of N-methylpriscol was due to liberation of adrenaline and it was found that the pressor action of N-methylpriscol was greatly reduced by adrenalectomy.

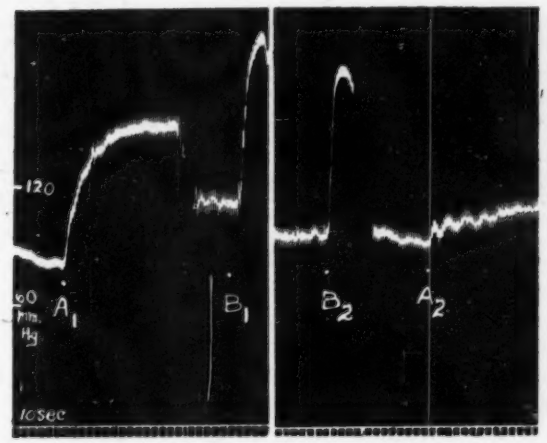


FIG. 6.—Record of blood pressure of spinal cat. At A_1, 20 mg. N-methylpriscol caused rise of blood pressure; at B_1, 10 μg. adrenaline had pressor action. Between B_1 and B_2 nicotine was injected until it no longer caused a rise (total injected 31.5 mg.). At B_2, 10 μg. adrenaline had a similar effect to that at B_1, but 20 mg. N-methylpriscol at A_2 had almost no action on blood pressure.

Since the pressor action of N-methylpriscol is largely due to the release of adrenaline, and since priscol abolishes the pressor action of adrenaline, it would be expected that priscol would modify the pressor action of N-methylpriscol. This proved to be so. In Fig. 7 a record of the nictitating membrane and of the blood pressure of a spinal cat is shown. At A, 20 μg. adrenaline was injected and, at B, 5 mg. N-methylpriscol. The latter caused a small prolonged contraction of the nictitating membrane. At C, 5 mg./kg. priscol was injected, and the nictitating membrane contracted strongly and persistently. At D, the same dose of adrenaline as was previously given at A caused a fall of blood pressure. The injection of 5 mg. N-methylpriscol at E now had a much smaller pressor action.

N-methylpriscol on the blood vessels.—When examined on the vessels of the rabbit's ear, doses of N-methylpriscol up to 1 mg. were without action.

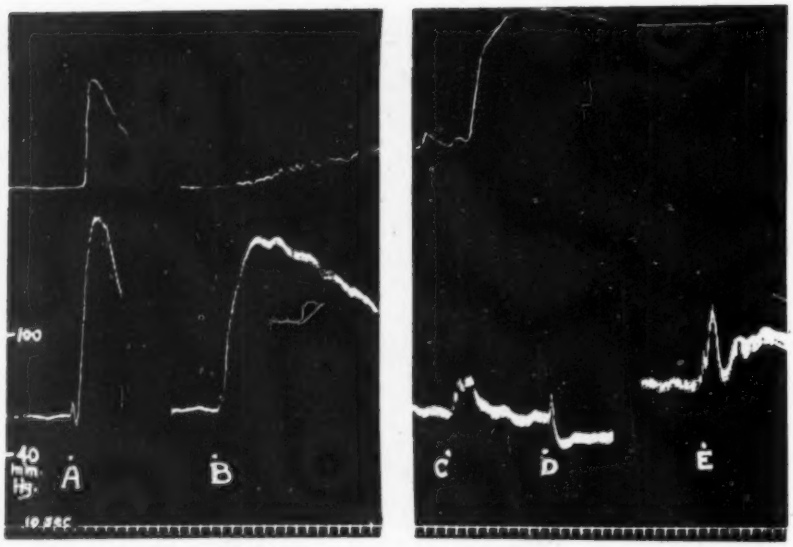


FIG. 7.—Upper record: contractions of nictitating membrane; lower record: blood pressure of spinal cat. At A, 20 μg. adrenaline caused contraction of nictitating membrane and rise of blood pressure; at B, 5 mg. N-methylpriscol caused slow contraction of nictitating membrane and rise of pressure; at C, 5 mg./kg. priscol caused powerful prolonged contraction of nictitating membrane; now at D, 20 μg. adrenaline was depressor, and 5 mg. N-methylpriscol at E had little pressor action.

N-methylpriscol on the heart.—On the cat heart perfused with Locke's solution through the coronary vessels, N-methylpriscol was found to have a stimulant action as shown in Fig. 8. At A, 0.5 mg. N-methylpriscol increased the rate from 120 to 206 per min., the coronary flow from 9.0 to 15.0 ml. per min. and the amplitude as shown in the figure. At B, 50 μg. nicotine produced a similar effect but of much shorter duration. Since N-methylpriscol caused a rise of blood pressure and increase of heart rate by a "nicotine-like" liberation of adrenaline from the adrenals, it was expected that its action upon the isolated heart would be another manifestation of the same "nicotine-like" property. But it was found that perfusion of the cat's heart with nicotine (2 mg. per ml.) abolished the stimulant action of nicotine, but not that of N-methylpriscol. In this respect N-methylpriscol resembles adrenaline and histamine, rather than nicotine, in its action upon the isolated heart.

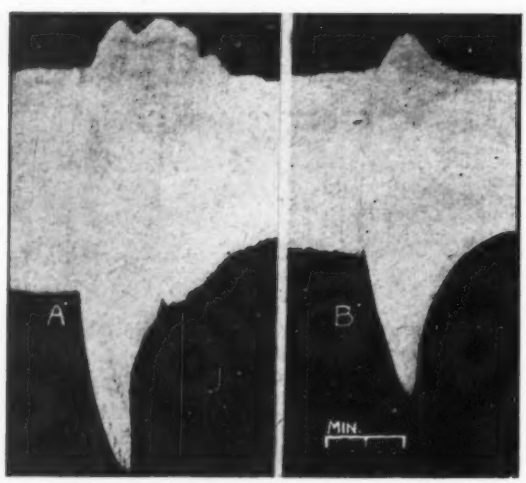


FIG. 8.—Record of contractions of isolated cat heart perfused by method of Langendorff. At A, 0.5 mg. N-methylpriscol increased force of contraction; heart rate from 120/min. to 206/min., and coronary flow from 9.0 ml./min. to 15.0 ml./min. At B, 50 μg. nicotine acid tartrate increased amplitude of contraction; heart rate from 128/min. to 160/min., and coronary flow from 8.6 ml./min. to 11.0 ml./min.

N-methylpriscol increased the rate and the amplitude of contraction of the isolated rabbit auricles; again it differed from nicotine, since tetraethylammonium iodide abolished the action of nicotine, but had no effect on that of N-methylpriscol; nor was the action of N-methylpriscol reduced by the antihistamine substance neoantergan.

N-methylpriscol did not affect the refractory period of the electrically-driven auricles even in a concentration as high as 10^{-4}, though this was enough to increase the spontaneous rate and the amplitude.

Relation to amidines.—The chemical structure of the imidazoline ring is related to that of the amidines. Dawes (1946b) showed that the latter potentiated the pressor action of adrenaline when injected into the splenic vein so as to pass through the portal system before entering the general circulation. Similar experiments were therefore performed with N-methylpriscol. Fig. 9 shows the blood pressure responses of a spinal cat to six injections of 20 μg. adrenaline; 5 mg. N-methylpriscol was mixed with the adrenaline in the

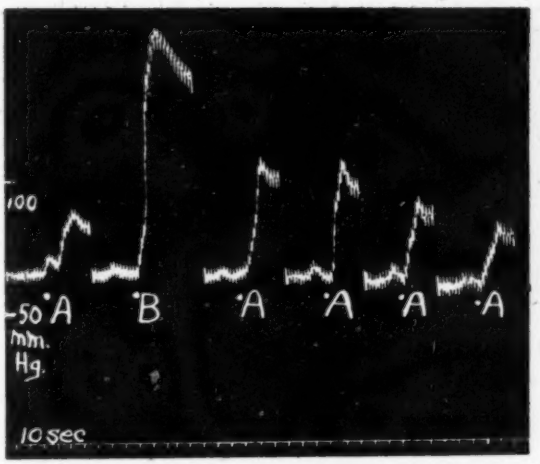


FIG. 9.—Record of blood pressure of spinal cat, all injections made into portal circulation through splenic vein. At each A, 20 μg. adrenaline produced rise of blood pressure; at B, 5 mg. N-methylpriscol was mixed with 20 μg. adrenaline and caused much greater rise of blood pressure.

second injection. The effect of the second injection was much greater than that of the first, and the succeeding injections progressively declined. Since the injection of 5 mg. N-methylpriscol alone had no effect on the blood pressure when given by this route, it clearly potentiated the action of adrenaline.

Smooth muscle.—The action of priscol was studied in the anaesthetised cat by inserting balloons in the oesophagus, duodenum and colon. In some experiments the vagi were stimulated below the heart, and in others the spontaneous intestinal movements were increased by the injection of eserine. Fig. 10 shows the record of the duodenal contractions; they were inhibited by adrenaline; a large contraction was induced by priscol (5 mg./kg.); 13 min. later another injection of adrenaline increased the contractions of the intestine, at the same time causing a fall of blood pressure. This reversal of the effect of adrenaline on the duodenum was not seen in other parts of the gastro-intestinal tract.

In the isolated duodenum of the rabbit priscol reduced the inhibitory action of both adrenaline and privine, but did not reverse their action.

Both priscol and N-methylpriscol were found to potentiate the action of acetylcholine on the guinea-pig ileum, while N-methylpriscol also potentiated the action of histamine in the guinea-pig ileum as shown in Fig. 11. It should, however, be observed that the spontaneous movements were also increased.

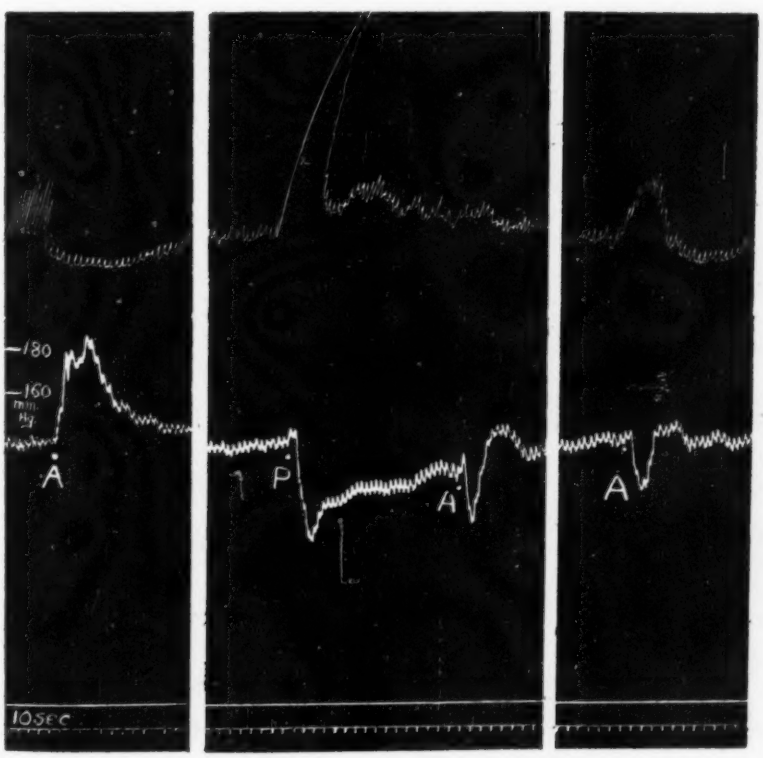


FIG. 10.—Upper record: contractions of duodenum recorded by intestinal balloon, water manometer and piston recorder; lower record blood pressure of cat under chloralose anaesthesia. At the first A, 10 μg. adrenaline inhibited duodenum and caused rise of blood pressure; at P, 5 mg./kg. priscol caused fall of blood pressure and large contraction of duodenum; 3 min. after priscol 10 μg. adrenaline, at second A, had depressor action but no effect on duodenum; 13 min. after priscol 10 μg. adrenaline, at last A, caused fall of blood pressure and *contraction* of duodenum.

FIG. 11.—Record of contractions of isolated guinea-pig ileum in Tyrode solution. All contractions caused by 0.15 μg. histamine except at arrow where 2 mg. N-methylpriscol was added to bath and left in for 30 sec. before the next 0.15 μg. histamine was added (at dot). Note the increased spontaneous activity after N-methylpriscol.

Skeletal muscle.—Priscol and N-methylpriscol potentiate the contractions of the frog's rectus abdominis caused by acetylcholine. When the cat's sciatic nerve is stimulated by maximal single shocks (16 per min.) the injection of either priscol or N-methylpriscol into the central end of the external iliac artery near the bifurcation caused a curariform depression of the contractions of the gastrocnemius muscle as shown in Fig. 12. The same effect was observed in the phrenic nerve diaphragm preparation of the rat (Bülbring, 1946), and in the frog gastrocnemius.

Superior cervical ganglion.—When the superior cervical ganglion of the cat was perfused with Locke's solution, and the contractions of the nictitating membrane were recorded, it was found that

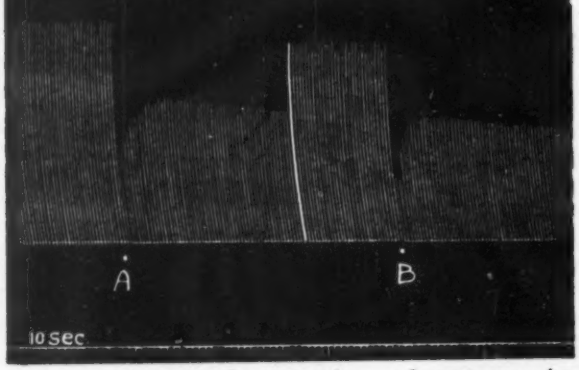


FIG. 12.—Record of contractions of gastrocnemius muscle of spinal cat caused by supra-maximal electrical stimulation (rate 16/min.) of sciatic nerve. At A, 8 mg. N-methylpriscol injected into external iliac artery had curariform action, and at B, 8 mg. priscol had same action.

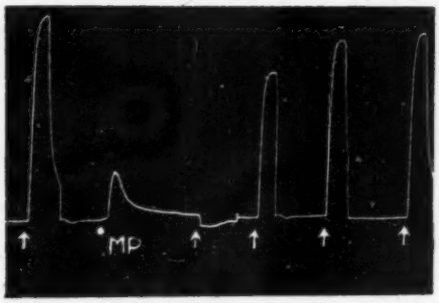


FIG. 13.—Record of contractions of nictitating membrane caused by supra-maximal electrical stimulation of pre-ganglionic fibres of cat's superior cervical ganglion perfused with warm Locke solution. At arrows stimulations lasting 15 sec. (given every 3 min.). At MP, 0.5 mg. N-methylpriscol caused contraction; it abolished the contraction due to the next stimulation, and reduced the second succeeding contraction.

both priscol and N-methylpriscol when injected into the perfusing fluid caused a contraction of the membrane. The contraction due to N-methylpriscol was more rapid in onset and in relaxation than that due to priscol, in contrast to the effect produced in the whole animal (Fig. 7). The injection of N-methylpriscol also abolished the effect of the next stimulation of the preganglionic fibres as shown in Fig. 13.

Gastric secretion.—When priscol was infused at a uniform rate into a vein it was found to cause a large output of gastric juice (collected by a wide cannula introduced through the pylorus in the stomach); this was observed in six cats anaesthetised with pentobarbitone. When N-methylpriscol was infused in four times the concentration, it failed to produce any secretion in two cats.

DISCUSSION

The observations described in this paper show that the reversal of the constrictor action of adrenaline by priscol, which is seen in the whole animal, can also be observed in the perfused vessels of the rabbit's ear. It is interesting to record that Rothlin (1925) found that ergotamine also would reverse the action of adrenaline in isolated vessels. The reversal of the pressor action of adrenaline is usually demonstrated in the whole animal; the fact that it also occurs in isolated vessels makes it more probable that it is due to a direct action of adrenaline itself, rather than to liberation of, for instance, histamine, as has been suggested by Staub (1946).

The observations here described show further that the introduction of a methyl group into the imidazoline ring of priscol changes the vascular action so that the peripheral vasodilatation is no longer seen ; instead a pressor action appears which can be attributed to a release of adrenaline by stimulation of the sympathetic ganglia. The pressor effect of N-methylpriscol is greatly reduced by the administration of either full doses of nicotine or of tetraethylammonium iodide, and also by the removal of the suprarenal glands. It is therefore very curious that though N-methylpriscol stimulates the isolated cat heart and the rabbit auricles, this does not appear to be a nicotine-like action, but may more properly be compared to the effect of adrenaline. In cardiac tissue the action of N-methylpriscol was in some respects similar to that of priscol.

While priscol converts the motor effects of adrenaline on the vessels to inhibitor effects, it reduces the inhibitory effects of adrenaline on the intestine, and in at least one instance converts them to motor. Thus after the injection of priscol which itself caused a contraction of the cat's duodenum (*in vivo*), the injection of adrenaline caused a contraction. The observations are very similar to those which have been made with ergotoxine and ergotamine, for these substances also convert the motor action of adrenaline on the vessels to an inhibitor action and as Planelles (1925) found, convert the inhibitor action on the intestine to a motor action. Priscol potentiated the action of acetylcholine on the intestine.

On skeletal muscle priscol and N-methylpriscol were alike in action. On the one hand, the stimulation of the frog rectus by acetylcholine was augmented, and on the other the contractions of the cat's gastrocnemius, when evoked by stimulation of the sciatic nerve, were diminished as by an injection of tubocurarine. This curariform action was also observed in the phrenic-diaphragm preparation of the rat. In the perfused superior cervical ganglion there was again evidence of a double action. Both priscol and N-methylpriscol, on injection into the perfusion fluid, stimulated the ganglion ; after the injection of N-methylpriscol, stimulation of the preganglionic fibres was temporarily ineffective. We therefore have in priscol and in N-methylpriscol substances which augment or imitate the action of acetylcholine in the sympathetic ganglion and in skeletal muscle ; in certain circumstances they have, as nicotine has, the opposite, curariform, action as well.

Perhaps the most striking conclusion to which we are driven by a study of the pharmacology of these substances is that they will not fit into any of the provisional classifications of drugs which in the past have proved so useful. This point can be more effectively illustrated by the following table :—

	Priscol	*N-Methylpriscol*
Blood pressure	Fall	Nicotine-like rise
Adrenaline action on blood pressure	Reversed	Unaffected
Adrenaline pressor effect after portal injection	?Unaffected	Augmented
Blood vessels	Vasodilatation	No direct effect
Adrenaline effect on vessels	Reversed	Unaffected
Smooth muscle	Abolishes adrenaline *in vitro* Reverses adrenaline *in vivo* (cat)	No effect on response to adrenaline
Spleen *in vivo*	Slight dilatation	Contraction
Cardiac muscle	Stimulated	Stimulated
Refractory period	Prolonged	Unaffected
Nictitating membrane	Strongly stimulated (ganglion removed)	Slight stimulation
Gastric secretion	Stimulated	Unaffected
Skeletal muscle	Like nicotine	Like nicotine
Superior cervical ganglion	Like nicotine	Like nicotine

It will be impossible to explain their mode of action until we have achieved more knowledge of the physiology of these tissues, but two points may provide a lead for subsequent investigations. The first is the structural relationship between the imidazolines, adrenaline and histamine, and the second is the observation that the reversal of the pressor action of adrenaline by priscol is dependent upon the relative concentrations of the two substances.

Summary

1. Reversal of the constrictor action of adrenaline by priscol, demonstrated in the whole animal, can also be observed in the perfused vessels of the rabbit's ear. The dilator action of adrenaline in these circumstances is more likely to be due to the action of adrenaline itself, rather than to the liberation of a substance such as histamine.

2. The introduction of an N-methyl group into priscol changes the vascular action so that, instead of peripheral vasodilatation, a pressor action is observed, which can be attributed to a release of adrenaline by stimulation of the sympathetic ganglia. The stimulating effect of N-methylpriscol on the isolated heart, however, does not appear to

be a nicotine-like action, but may be compared to the effect of adrenaline. In cardiac tissue the action of priscol is, in some respects, similar to that of N-methyl derivative.

3. Priscol reduces the inhibitory effects of adrenaline on the intestine, and in at least one instance converts them to motor. Both priscol and N-methylpriscol potentiate the action of acetylcholine on the intestine.

4. In the sympathetic ganglion and in skeletal muscle priscol and N-methylpriscol augment or imitate the actions of acetylcholine, and in certain circumstances they have the opposite, curariform, action as well.

5. A study of the pharmacology of these substances indicates that they cannot be fitted into the provisional classifications of drugs which have hitherto been so valuable.

The N-methylpriscol was very kindly supplied by Dr. W. F. Short, Director of the Chemical Research Laboratories of Messrs. Boots Pure Drug Co., Ltd. I wish to thank Prof. J. H. Burn and Dr. G. S. Dawes for the guidance and advice which they have given throughout this study.

References

Barger, G., and Dale, H. H. (1910). *J. Physiol.*, **41**, 19.
Beyer, K. H. (1946). *Physiol. Rev.*, **26**, 169.
Bülbring, E. (1946). *Brit. J. Pharmacol.*, **1**, 38.
Chess, D., and Yonkman, F. F. (1945). *Fed. Proc.*, **4**, 114.
Dale, H. H., and Schuster, E. H. J. (1928). *J. Physiol.*, **64**, 356.
Dawes, G. S. (1946a). *Brit. med. J.*, **1**, 43.
Dawes, G. S. (1946b). *Brit. J. Pharmacol.*, **1**, 21.
Gaddum, J. H., and Kwiatkowski, H. (1938). *J. Physiol.*, **94**, 87.
Hartmann, M., and Isler, H. (1939). *Arch. exp. Path. Pharmak.*, **192**, 141.
Hermann, H., Jourdan, F., and Bonnet, V. (1941). *C.R. Soc. Biol.*, **135**, 1653.
Meier, R., and Müller, R. (1939). *Schweiz. med. Wschr.*, **69**, 1271.
Planelles, J. (1925). *Arch. exp. Path. Pharmak.*, **105**, 38.
Rothlin, E. (1925). *Klin. Wschr.*, **4**, 1437.
Staub, H. (1946). *Helv. Physiol. Acta*, **4**, 539.

Brit. J. Pharmacol. (1948), **3**, 263.

ANALYSIS OF ANTIMITOTIC ACTION OF CERTAIN QUINONES

BY

E. FRIEDMANN, D. H. MARRIAN, AND (MRS.) I. SIMON-REUSS

From the Department of Radiotherapeutics, University of Cambridge

(Received February 28, 1948)

The study of mitotic inhibition in experiments dealing with toxicological and pharmacological problems has been used by Dustin (1934) with great success. He worked with normal tissues and tested antimitotic activities *in vivo*. The very valuable method of tissue cultures has been applied by Ludford (1936) for the same purpose. Other biological materials for the investigation of physiological processes on the same lines have been adopted by Töndury (1941), Lehmann (1942) and Lüscher (1946): Töndury worked with *Triton eggs*, Lehmann (1945, 1946) and later Huber (1947) with the eggs of *Tubifex*; Lüscher investigated regeneration in *Xenopus larvae* under the influence of colchicine. Barber and Callan (1943) studied the effect of cold and colchicine in mitoses of the newt.

From the beginning it was clear that the study of mitotic inhibitors may well pave the way for a chemotherapeutic approach to the cancer problems (Dustin, 1939). The great variety of antimitotic substances and the lack of any chemical connexion between many of them seemed to be a serious obstacle. Furthermore nearly all the potent antimitotics are poisonous to normal cells at concentrations at which they exert antimitotic action on tumour cells, in this way restricting the use of antimitotics to external application on surface tumours. Broderson (1943) has given evidence that it is possible by this method to control the growth of malignant tumours of the skin; he used colchicine, whose strong activity as a mitotic inhibitor was well known.

Nevertheless these difficulties might be overcome. Mitchell (1942) and v. Euler and Hevesy (1942) have shown that therapeutic doses of *x*- and gamma-radiations inhibit the synthesis of thymonucleic acid in proliferating normal and malignant cells. Substances able to interfere with the metabolism of nucleoproteins ought therefore to be endowed with antimitotic properties, and conversely some mitotic poisons should be able to interfere with the metabolism of nucleoproteins; thus the mechanism by which disturbances in the metabolism of nucleoproteins are induced becomes a possible common denominator for the various antimitotic substances even when they are chemically unrelated. To give an example: according to Kopac (1945) stilbamidine blocks mitosis in some neoplastic cells; it dissociates protamine-ribonucleates, releasing the protamine and simultaneously trapping the ribonucleate complex by the formation of an insoluble stilbamidine-ribonucleate. Nucleoproteins, therefore, can be inactivated by low concentrations of stilbamidine. Lettré and his collaborators (1946) found that substances of the type R—Hg—X, where R is an alkyl or an aryl residue, X an inorganic or organic anion or a phenol, are mitotic poisons. They explain the antimitotic activity of these compounds by pointing out that nucleic acids and nucleoproteins are able to combine by salt or complex formation with the organometallic compounds to give insoluble products. The similarity in the mechanism by which stilbamidine and the organometallic compounds disclose their antimitotic properties is easily seen. They belong to the same physiological group of antimitotics, although they are chemically as dissimilar as possible.

As mentioned above, the second great difficulty for the therapeutic utilization of antimitotics is the fact that the concentration at which they display the antimitotic activity is nearly always within the range of their toxicity for normal cells. Two ways of overcoming this difficulty have been investigated. The molecule may be changed chemically until it has the required properties, either by investigating the corresponding homologous series or by introducing new radicals in the hope of improving the therapeutic index. The other way is to

cover the active group by substitution. The work of the cell consists then in releasing the active principle within the cell. This is possible if the cell contains enzymes capable of splitting off the substituent. The choice of the substituent is therefore of primary importance. In view of the role which phosphoric acid plays in the synthesis of nucleic acid and the ease of its transfer in the processes supplying the necessary energy for these and other reactions, the phosphate residue stands out as the most promising substituent. In 1946 Mitchell started to study the therapeutic effects of the diphosphate of 2-methyl-1:4-naphthohydroquinone, in combination with radiation, in cancer patients and later gave evidence in collaboration with Simon-Reuss (1947) that this diphosphate produces mitotic inhibition in chick fibroblasts and in some human carcinomata, with potentiation of the mitotic inhibitory effects of *x*-radiation under suitable conditions in the tissue cultures. The mitotic inhibition produced by the unsubstituted 1:4-naphthohydroquinone was discovered by Lehmann (1942), who found it active at a concentration of 10^{-7}; the same degree of activity was displayed by the corresponding quinone. Lehmann worked with *Tubifex eggs*. Meier and Allgöwer (1945) and Meier and Schär (1947) obtained similar results with chick fibroblast cultures.

In our own experiments we followed two lines of research. We tried to ascertain the chemical group in the quinone molecule, common to the antimitotics under investigation and responsible for their chemotherapeutic activity. We tried further to get some evidence which would allow us to correlate our results with physiological processes, the disturbance of which may cause interruption of growth, as shown by mitotic inhibition.

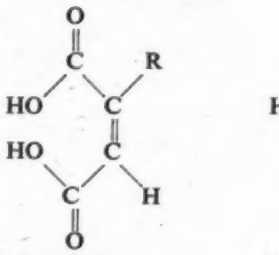
The present communication is the first of a series of papers dealing with these problems.

Methods

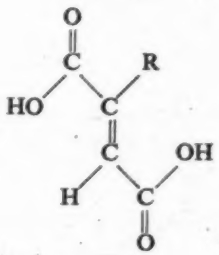
All the experiments were carried out on tissue cultures of chick fibroblasts. The tissues used were taken from the sclerotic and choroid of a 10–11-days-old chick embryo. The medium consisted of equal parts of fowl plasma and of 15 per cent embryo extract. The usual hanging drop technique was applied. Two groups of six cultures of the 4th passage were selected and matched for experimental purposes and controls. The compound to be tested was dissolved in Tyrode solution and the *p*H adjusted to 7.4. The solution was suitably diluted with Tyrode and sterilized by filtration through Seitz filter. Sterilization by boiling in Tyrode was avoided because the boiling of maleic and fumaric acids in Tyrode solution gave unreproduceable results. The diluted solution of the substance was then added to the embryonic extract. The final molar concentrations appear in Table I. The cultures were fixed and stained with Heidenhain's haematoxylin at given intervals and mitotic counts were carried out.

Experimental

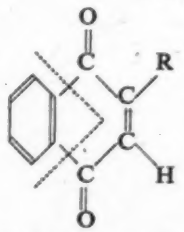
Many chemical properties of the quinones are centred in the non-benzenoid part of the molecule and the same may apply to the physiological activities. Thus the question arises whether the benzene ring of the naphthoquinones (V) is necessary for the mitotic inhibition induced by them. Maleic acid (I), its methyl derivative, citraconic acid (II), and their *trans*-isomers, fumaric acid (III), and mesaconic acid (IV), were therefore investigated.



I, R = H
II, R = CH_3



III, R = H
IV, R = CH_3



V, R = H or CH_3

I. *Maleic acid*

Pure maleic acid was prepared by hydrolysis of maleic anhydride. The results obtained with maleic acid are given in Table I and show that maleic acid has strong antimitotic properties. At a concentration of 0.5×10^{-6} molar the inhibition was 33.6 and 54.2 per cent in two experiments. The mitotic inhibition increased with rising concentration. The increase follows roughly a logarithmic graph, when plotted against the log 10 of the concentration (Fig. 1). The phase distribution calculated in percentage of the total mitoses gives no decisive information. Microscopically the picture common to all substances with antimitotic activity was observed: clumped metaphases and undivided telophases were seen in all phases; fragmentation of chromosomes occurred in all phases, but mainly in anaphases. At higher concentrations

TABLE I

TISSUE CULTURES: CHICKEN FIBROBLASTS, HANGING DROP METHOD, 4TH PASSAGE, 24 HOUR CULTURES, FIXED IN SUSA, STAINED IN HEIDENHAIN'S HAEMATOXYLIN

Exp.	Molar conc.	Mitoses as % of mitoses of controls	Per cent inhibition	Phase distribution in % of mitoses			
				Prophase	Metaphase	Anaphase	Telophase
MALEIC ACID (10,795 mitotic cells investigated)							
1.	Controls	—	—	11.8	38.1	3.4	46.8
2.	0.5×10^{-6}	45.8 ± 3.8	54.2	17.8	30.4	3.5	51.2
3.	1×10^{-6}	32.6 ± 5.0	67.4	15.7	21.3	3.8	59.2
4.	2×10^{-6}	45.5 ± 8.2	54.5	12.3	46.0	6.1	35.6
5.	Controls	—	—	16.8	29.5	4.4	49.2
6.	0.5×10^{-6}	66.4 ± 5.2	33.6	18.1	39.4	3.0	39.6
7.	2×10^{-6}	15.7 ± 3.4	84.3	24.9	25.8	3.0	46.4
8.	4×10^{-6}	6.0 ± 1.4	94.0	18.6	31.4	3.9	46.1
9.	Controls	—	—	22.5	25.6	5.9	46.0
10.	1×10^{-6}	35.5 ± 2.2	64.5	19.0	44.6	7.8	28.2
11.	2×10^{-6}	32.4 ± 6.8	67.6	18.9	47.6	7.2	26.2
12.	3×10^{-6}	23.4 ± 3.1	76.6	18.4	38.5	4.5	38.5
CITRACONIC ACID (5.449 mitotic cells investigated)							
1.	Controls	—	—	21.1	29.3	7.5	42.1
2.	3×10^{-6}	98.4 ± 2.4	—	21.9	33.7	4.3	40.0
3.	Controls	—	—	13.5	37.5	9.5	39.5
4.	3×10^{-6}	97.3 ± 6.0	—	13.4	38.6	9.9	38.1
FUMARIC ACID (7,841 mitotic cells investigated)							
1.	Controls	—	—	27.1	28.7	1.8	41.8
2.	1×10^{-4}	105.4 ± 1.8	—	26.9	30.9	4.5	37.7
3.	3×10^{-5}	87.3 ± 13.8	—	28.5	22.4	4.7	44.5
4.	Controls	—	—	19.7	29.5	6.1	44.7
5.	3×10^{-6}	84.8 ± 6.9	15.2	21.9	25.9	9.9	42.3
6.	2×10^{-6}	87.9 ± 7.10	—	22.9	36.1	7.7	33.2
7.	1×10^{-6}	99.7 ± 10.0	—	21.0	34.6	5.5	38.9
MESACONIC ACID (3,374 mitotic cells investigated)							
1.	Controls	—	—	18.4	33.9	4.4	43.4
2.	5×10^{-6}	100 ± 3.5	—	30.4	15.7	3.3	50.7
3.	3×10^{-6}	100 ± 6.9	—	19.4	19.9	4.9	55.7
4.	1×10^{-6}	100 ± 3.3	—	16.4	15.4	5.3	62.9
TETRASODIUM 1:4-NAPHTHOHYDROQUINONE DIPHOSPHATE (5,838 mitotic cells investigated)							
1.	Controls	—	—	28.0	34.7	1.2	36.0
2.	1×10^{-6}	21.5 ± 4.8	78.5	3.4	86.2	0.7	9.7
3.	5×10^{-7}	35.7 ± 5.8	64.3	5.2	86.2	0.3	8.3
4.	Controls	—	—	16.9	22.9	1.7	58.8
5.	3×10^{-8}	8.4 ± 2.5	91.6	7.0	69.0	1.4	22.5
6.	1×10^{-9}	19.4 ± 3.5	80.6	10.6	55.9	5.0	28.6
7.	5×10^{-9}	41.0 ± 9.1	59.0	21.9	38.0	5.8	34.2
8.	Controls	—	—	19.8	23.0	2.3	54.9
9.	3×10^{-9}	49.1 ± 6.1	50.9	20.7	24.9	4.2	50.2
10.	1×10^{-9}	77.7 ± 8.9	22.3	16.9	35.8	2.7	44.7
11.	5×10^{-10}	86.2 ± 9.7	13.8	19.5	28.6	3.8	48.1
1:4-NAPHTHOHYDROQUINONE MONO-HYDROGEN SUCCINATE (7,557 mitotic cells investigated)							
1.	Controls	—	—	20.6	38.7	5.7	35.0
2.	1×10^{-9}	103 ± 10.4	—	21.7	30.1	4.7	43.4
3.	3×10^{-9}	105 ± 10.6	—	22.7	29.0	4.9	43.6
4.	5×10^{-9}	104 ± 9.3	—	22.5	30.0	4.4	43.1
5.	Controls	—	—	13.5	36.9	3.9	45.8
6.	1×10^{-6}	103 ± 2.4	—	14.4	43.9	5.7	36.0
7.	3×10^{-6}	102.5 ± 2.1	—	14.7	42.7	6.0	36.6
8.	5×10^{-6}	99.3 ± 2.3	—	14.0	44.9	4.6	36.5

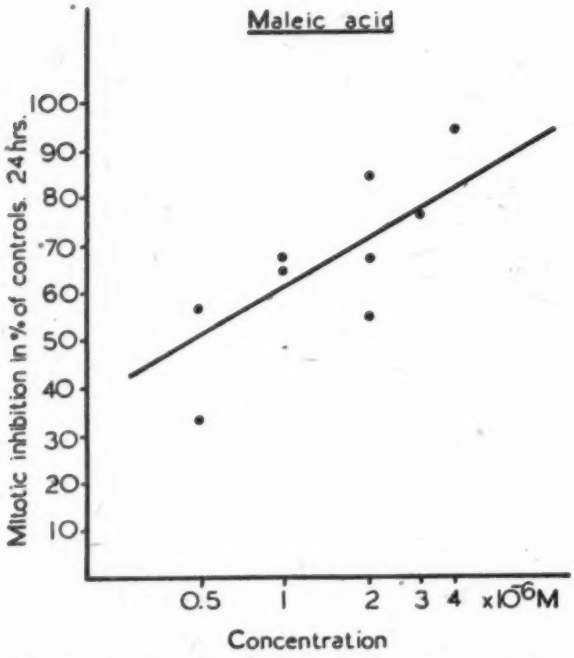


FIG. 1.—Mitotic inhibition plotted as percentage of controls against the logarithm of the concentration of maleic acid.

(3 and 4 × 10^{-6} *M*) the cultures showed but little outgrowth, the resting cells were flattened out and some exploded cells could be seen.

II. *Citraconic acid*

The methyl derivative of maleic acid, citraconic acid, showed no mitotic inhibition. There was no difference in the phase distributions compared with the controls (Table I). Microscopically a few clumped metaphases were to be seen but no other abnormalities.

III. *Fumaric acid*

Table I shows that fumaric acid induces no mitotic inhibition. In the phase distribution a slight increase only in anaphase is to be noted. Microscopically all concentrations showed slight mitotic disturbances: clumped metaphases and chromosome breakages in late metaphases and anaphases were seen; only the telophases seemed not to be affected. Microscopical pictures were found which resembled those obtained with maleic acid. Abnormal cells were present, and the proportion in which they were present was very near that observed in normal tissue cultures, viz., 5 per cent. (Found abnormal mitoses in per cent of the mitoses in the experiment at 1 × 10^{-6}*M*: 6.6 ± 0.7 per cent from 377 cells in mitosis; at 2 × 10^{-6}*M*: 9.2 ± 1.72 per cent from 338 cells in mitosis; at 3 × 10^{-6}*M*: 4.6 ± 0.97 per cent from 305 cells in mitosis.)

IV. *Mesaconic acid*

Mesaconic acid (methyl fumaric acid) (Table I) showed no mitotic inhibition and no abnormal cells were found. The phase distribution seemed to indicate an increase in telophase in the lower concentrations which would have to be investigated further.

V. *1 : 4–Naphthohydroquinone diphosphate*

Lehmann (1942) has already shown that 1 : 4–naphthoquinone is a stronger antimitotic than its 2–methyl derivative. He worked with *Tubifex eggs*. Meier and Allgöwer (1945) and Meier and Schär (1947) tested the substance in tissue cultures and described the cytological pictures obtained.

As quinones and hydroquinones have apparently the same degree of antimitotic activity (Lehmann, 1942) we prepared the previously unknown 1 : 4–naphthohydroquinone diphosphate in order to ascertain quantitatively its activity in tissue cultures.

Table I gives the results. It will be seen that 1 : 4–naphthohydroquinone diphosphate is an extremely powerful inhibitor of mitosis. Compared with the results calculated by Huber (1947) for the antimitotic activity of 1 : 4–naphthoquinone a remarkable agreement is to be noted: Huber found 1 : 4–naphthoquinone active at 0.7 × 10^{-9}*M* for *Tubifex eggs*, whereas our experiments on chick fibroblasts show threshold activity of 1 : 4–naphthohydroquinone diphosphate at 0.5 × 10^{-9}*M*. Compared with the 2–methyl derivative, tested by Mitchell and Simon-Reuss (1947), the activity of

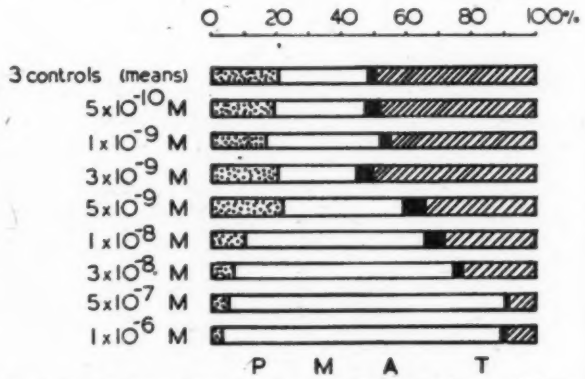


FIG. 2.—Tetrasodium 1 : 4-naphthohydroquinone diphosphate. Phase distribution as percentage of total mitoses: normal from 5 × 10^{-10} to 3 × 10^{-9}*M*, increase of metaphases from 5 × 10^{-9}*M*.

the new methyl free derivative is more than 1,000 times greater. With rising concentration the activity of the diphosphate increases. From $5 \times 10^{-10} - 1 \times 10^{-5} M$ the increase follows a logarithmic line. The phase distribution (Fig. 2) is nearly normal from $5 \times 10^{-10} M - 3 \times 10^{-9} M$, but at $5 \times 10^{-9} M$ the metaphases are already increased. This increase grows continuously with rising concentration until at $5 \times 10^{-7} M$ 86 per cent of the total mitoses are in metaphase. In the whole range of $10^{-5} M$, the range of its mitotic inhibition, no toxic effect was observed, although the general picture found in mitotic disturbances was well in evidence: clumped metaphases, in all phases some chromosome breakages, some enlarged metaphases and undivided telophases. Toxic effects began to appear at $10^{-5} M$, vacuolized and exploded cells being found. Abnormal cells were investigated for the concentration range $0.5 \times 10^{-9} - 1 \times 10^{-5} M$.

VI. *1 : 4–Naphthohydroquinone mono-hydrogen succinate*

This substance was prepared in order to see whether the phosphate residue in the 1 : 4–naphthohydroquinone diphosphate could be replaced by another acidic group.

Table I shows that 1 : 4–naphthohydroquinone mono-hydrogen succinate had no effect on mitosis. The phase distribution seemed to be slightly affected, but the figures allowed no clear interpretation. Microscopical investigation showed the absence of abnormal mitoses.

Discussion

In this paper we have used tissue cultures as a method of approach to the chemotherapy of growth inhibition. Mitotic inhibition, phase distribution, and the cytological picture have all been studied as indices of the effects exerted on tissue growth by the different agents. The picture disclosed by the three parts of this analysis may seem to give a reliable basis for the chemotherapeutic evaluation of the substances tested; this is obviously only true within certain limits, although the results given in the three parts of this analysis have been obtained by quantitative methods.

The study of phase distribution alone for instance gives little reliable information. Maleic acid, a strong inhibitor of mitosis, shows no appreciable change in phase distribution (Table I). Furthermore disturbances in phase distribution may precede mitotic inhibition at lower concentrations (unpublished data) or they may follow mitotic inhibition after the peak of the inhibition has nearly been reached (1 : 4–naphthohydroquinone diphosphate; Table I, experiment 7), or one set of experiments may display a shift of the phases in one direction, another set in the opposite direction (Table I, 1 : 4–napthohydroquinone mono-hydrogen succinate).

Mitotic disturbances, as shown by the occurrence of abnormal cells, without mitotic inhibition and without alteration in phase distribution have not been prominent with the substances examined in this paper; but they occur, as shown by v. Möllendorff (1938), and the slight indications of such a possibility, encountered in the study of fumaric acid, convey a warning which cannot be dismissed The quantitative determination of abnormal mitoses in connection with mitotic inhibition may provide useful chemotherapeutic information. A special investigation is planned to elaborate these relations. The figures given for mitotic inhibition seem to have a greater individual importance than those for phase distribution. They are the result of many mitotic processes and represent an average expression of them. Their fluctuations, recorded statistically, are, as a rule, within reasonable limits and allow their use as a quantitative expression of mitotic inhibition. Thus the positive statement of mitotic inhibition of a definite degree for a given concentration is fairly safe. The negative statement, no mitotic inhibition, based only on the absence of mitotic inhibition, may be erroneous.

Taking mitotic inhibition to express our results the experiments can be summarized as follows:

Mitotic inhibition	I. Maleic Acid.	II. Citraconic acid.	III. Fumaric acid.	IV. Mesaconic acid.
Mol/1:	5×10^{-7}	None.	None.	None.

Mitotic inhibition	V. 1 : 4-naphthohydroquinone diphosphate.	VI. 1 : 4-naphthohydroquinone mono-hydrogen succinate.	2-methyl-1 : 4-naphthohydroquinone diphosphate. (Mitchell and Simon-Reuss)
Mol/1:	1×10^{-9}	None.	3×10^{-6}

The results show that the ring structure of the quinones is not necessary for inducing antimitotic activity. Maleic acid (I) which contains the aliphatic part of the quinone molecule is a strong antimitotic. In this connection it may be remembered that Brunschwig *et al.* (1946) were able to show that the growth of cancer in rats can be retarded by maleic acid.

Introducing a methyl group into maleic acid gives citraconic acid (II), which does not show the antimitotic properties of maleic acid.

The importance of the *cis*-configuration of maleic acid is shown by the behaviour of fumaric acid (III) and mesaconic acid (IV), the *trans*-

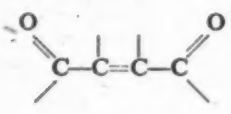
isomers of maleic and citraconic acids. Fumaric acid exerts no mitotic inhibition, in agreement with the results obtained by Pomerat and Wilmer (1939); mesaconic acid was also completely inactive.

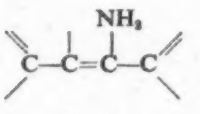
A methyl group introducted into maleic acid has obviously a dystherapeutic effect. The same is true in the 1 : 4–naphthoquinone series. The methyl free 1 : 4–naphthohydroquinone diphosphate belongs to the most powerful antimitotics obtained so far. Compared with colchicine and taking the figures given by Huber (1947) for its antimitotic action on *Tubifex eggs* as basis ($8.41 \times 10^{-6}M$) it is approximately 15 times more active than colchicine.

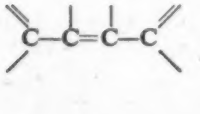
The importance of phosphorylation, inaugurated in this series by Mitchell and Simon-Reuss (1947) with the examination of the diphosphate of 2–methyl–1 : 4–naphthohydroquinone, is again apparent. Introducing succinic acid instead of phosphoric acid in 1 : 4–naphthohydroquinone gave 1 : 4–naphthohydroquinone mono-hydrogen succinate (VI) which was completely inactive. The role of the phosphorylation will be discussed in another paper of this series.

The present investigation discloses clearly the unit which maleic acid and the quinones have in common. We find in both groups the same uninterrupted system of three conjugated double bonds in which the second double bond is activated by the two adjacent C=O groups. The experimental evidence, so far obtained, seems to suggest that the unit O=C–CR=CH–C=O, R=H, in maleic acid and R=H or CH_3 in the quinones, is connected with the antimitotic activity of this group.

This unit is closely related to stilbylamine, which Lettré (1946) has shown to be the active principle in the great colchicine group, basing his deduction on the Windaus formula for colchicine, although not disregarding the alternative structure put forward by Cook *et al.* (1944) and by Dewar (1945). It is also related to stilbene, enclosed in the stilboestrols and investigated in tissue cultures by von Möllendorff (1939) and by Lettré and Albrecht (1941 and 1943).


in quinones,


in the colchicine group,


in the stilboestrols.

Chemical Section

Preparation of 1 : 4 - naphthohydroquinone diphosphate and its salts. Dibarium salt.—A stream of nitrogen was passed into a flask containing phosphorous oxychloride (9 c.c.) in pure dry pyridine (60 c.c.). The contents were stirred in ice during the dropwise addition over 45 minutes of an ice-cold solution of 1 : 4-naphthohydroquinone (3.5 g.) in pyridine (90 c.c.). After final addition, the mixture was stirred in ice for 30 minutes and then evaporated *in vacuo* under nitrogen at 50°. When the residue was almost dry, pyridine (50 c.c.) was added and the evaporation repeated. The residue was left overnight *in vacuo* over sulphuric acid. Water (200 c.c.) was then added, the whole well shaken and left at room temperature for 1 hour. Hyflo supercel (*ca.* 1 g.) was added, the liquid filtered under reduced pressure, and the pale yellow filtrate made just alkaline to phenolphthalein by addition of a saturated solution of barium hydroxide (*ca.* 1 litre). The small precipitate was filtered off after addition of Hyflo and the filtrate evaporated *in vacuo* under nitrogen at 70° to about 500 c.c. The concentrate was filtered through a layer of Hyflo on a Buchner funnel and the filtrate added slowly with shaking to alcohol (500 c.c.). The flocculent precipitate was left for 36 hours at room temperature in the dark after displacing air with nitrogen. The product formed a gel which was centrifuged off, washed once with 50 per cent aqueous alcohol, once with alcohol, and finally with ether. The residue was dried *in vacuo* over silica gel. Crude yield 13.3 g. colourless amorphous solid. The salt appears to decompose when heated in solution.

Tetracyclohexylamine salt.—A solution of *cyclo*hexylamine (5 c.c.) in water was titrated with *N*. sulphuric acid till a *p*H of 3.8 was reached (43.5 c.c. 1.006*N*. sulphuric acid required). This solution was diluted to 100 c.c. with distilled water.

Dibarium 1 : 4-naphthohydroquinone diphosphate (3.2435 g., crude) was dissolved almost completely in water (100 c.c.) and titrated with the above *cyclo*hexylamine sulphate solution till only a trace of free barium ions remained (as tested for by an aqueous solution of sodium rhodizonate). This required 44.5 c.c.; if the barium salt had been pure, 53.5 c.c. would have been needed. Hyflo supercel was added, the precipitated barium sulphate filtered off through a layer of Hyflo and the filtrate evaporated to dryness *in vacuo* over nitrogen at 60°. The gummy residue was dissolved in methyl alcohol (25 c.c.), decolorized with charcoal, and evaporated to about 10 c.c. Ether was added till a slight opalescence appeared. On standing overnight prisms of the *tetracyclohexylamine salt of 1 : 4-naphthohydroquinone diphosphate* separated which were filtered off and washed with methyl alcohol–ether (1 : 1), then with ether, and dried; 2.1 g. colourless prisms (yield 50 per cent) m.p. 193.5–194° (decomp. after sintering at 192°). For analysis, a specimen was recrystallized from methyl alcohol, m.p. unchanged. (Found: C, 54.6; H, 9.0; N, 7.45 per cent. $C_{10}H_{16}O_6P_2.4C_6H_{13}N.2H_2O$ requires C, 54.2; H, 8.8; N, 7.44 per cent).

Tetrasodium salt.—The tetra*cyclo*hexylamine salt of 1:4-naphthohydroquinone diphosphate (1.7330 g.) in water (25 c.c.) was treated with the theoretical amount of sodium hydroxide solution (96.5 c.c. of 0.0956*N.*), the resulting solution filtered through a sintered glass funnel, a little redistilled hexyl alcohol added, and the whole evaporated *in vacuo* over nitrogen at 70°. The colourless oily residue gradually crystallized when left *in vacuo*. The material was ground up with alcohol in an agate mortar, filtered off and dried. The *tetrasodium salt of 1 : 4-naphthohydroquinone diphosphate* crystallized in colourless prisms; 0.8 g. (yield 85 per cent). (Found, in material dried at 50°: C, 24.5; H, 3.2. Loss in weight at 180°, 19.0 $C_{10}H_6O_8P_2Na_4.5H_2O$ requires C, 24.1; H, 3.2. Loss of $5H_2O$ requires 18.1 per cent). The salt is soluble in cold water to form a neutral solution, and forms a gel on addition of organic solvents.

1 : 4-Naphthohydroquinone diphosphate.—(*a*) From the tetrasodium salt. Tetrasodium salt of 1:4-naphthohydroquinone diphosphate (0.45 g.) was treated with *ca.* 5*N.* hydrochloric acid (1.1 c.c.) and water (5 c.c.). The solution was evaporated to dryness under reduced pressure, and the residue well extracted with hot alcohol (total 15 c.c.). The extract was filtered, treated with a little Hyflo and filtered through a little Hyflo. The filtrate was evaporated to dryness *in vacuo* under nitrogen at room temperature and the residue left to solidify.

The resulting solid, admixed with some oil, was dried on a porous tile, ground up with dry ethyl acetate, filtered off and dried. *The compound* formed a colourless partially crystalline solid, m.p. 214–215° (decomp.); 0.2 g. (yield 68 per cent). (Found: C, 34.6; H, 3.3. Equiv. 84.5. Loss in weight at 180° with some decomposition, 9.0.* $C_{10}H_{10}O_8P_2.1\frac{1}{2}H_2O$ requires C, 34.6; H, 3.7. Equiv. 86.8. Loss of $1\frac{1}{2}H_2O$ requires 7.4 per cent).

(*b*) From crude dibarium salt. The dibarium salt (7.879 g.) was stirred in water (200 c.c.) till solution was almost complete and *N.* sulphuric acid added till only a trace of free barium ions remained, the precipitated barium sulphate filtered off through a layer of Hyflo, and the colourless filtrate evaporated to dryness under reduced pressure, leaving 1.9 g. of waxy solid. This solid was dissolved in alcohol (150 c.c.), filtered through Hyflo, and allowed to evaporate at room temperature *in vacuo*. The residue crystallized, m.p. 210–212° (decomp.) (equiv. 77.3). The alcohol treatment was repeated and when most of the alcohol had evaporated, a little ethyl acetate was added. The *1 : 4-naphthohydroquinone diphosphate* partially crystallized in colourless prisms on standing, m.p. 214–215° (decomp.). (Found, in material dried at 80°: C, 36.9; H, 3.6 per cent. Equiv. 79.6. $C_{10}H_{10}O_8P_2.\frac{1}{2}H_2O$ requires C, 36.5, H, 3.4 per cent. Equiv. 82.3.)

The tetra*cyclo*hexylamine salt can also be prepared from the free acid in water by addition of excess *cyclo*hexylamine. The residue after evaporation crystallizes from methyl alcohol, m.p. 193.5–194° (decomp.) identical with the material prepared from the dibarium salt.

1 : 4-Naphthohydroquinone mono-hydrogen succinate.—1:4-Naphthohydroquinone (1 g.) and maleic anhydride (6 g.) were carefully mixed and the mixture heated under nitrogen for two hours at 160°. The molten mass was collected, powdered, and extracted with hot ether (400 c.c.) in portions of 50 c.c. The ether extract was filtered after 24 hours from undissolved anhydride (3.3 g.) and extracted with 1 per cent sodium carbonate (45 c.c.) in portions of 4×10 c.c. and 1×5 c.c. The first extracts were acid, the last extract was amphoteric against litmus. The combined 1 per cent sodium carbonate extracts remained clear on acidifying and were discarded. A second extraction with 5 per cent sodium carbonate followed (90 c.c. in portions of 20, 20, and 5×10 c.c.). The second extract, at once acidified with 5 *N.* sulphuric acid (15 c.c.), gave an oily, reddish precipitate which solidified quickly. The precipitate was dissolved in ether. The red brownish ethereal solution was washed with water, dried over anhydrous sodium sulphate, decolorised with charcoal, and brought to dryness. The slightly coloured, crystalline residue (0.7 g.) was extracted with hot benzene (20 c.c.) which removed the coloured impurities completely. Yield 0.6 g., m.p. 175–176° (decomp.). Recrystallization from ether/*n*-hexane gave a product melting at 171°, which was again recrystallized from methanol/water. It was finally obtained in transparent, irregularly shaped plates, single or arranged in rosettes of tilted plates, m.p. 167° (decomp.). Dissolved in dilute alcohol, the substance gives a purple colour reaction with aq. ferric chloride. (Found: C, 64.5; H, 4.35. Equiv. 251. $C_{14}H_{12}O_5$ requires: C, 64.6; H, 4.65 per cent. Equiv. 260.)

1 :4-Naphthohydroquinone bis-hydrogen succinate.—1:4-Naphthohydroquinone (0.5 g.) and maleic anhydride (3 g.) were brought to reaction as described above. The molten mass was poured into water (50 c.c.). The undissolved material was powdered and re-extracted with water (50 c.c.) for one hour. The residue was collected, washed and dried *in vacuo*. The dried substance (0.5 g.) was dissolved in ether (75 c.c.) by heating. The slightly cloudy solution became clear after being gently treated with norite. The norite was removed by filtration and the filtrate concentrated until crystals began to appear. The crystallization was completed by adding *n*-hexane in the usual way. The collected crystals (0.4 g., m.p. 137°) were recrystallized from hot ether (180 c.c.), discarding small amounts of ether insoluble material. The ethereal solution was concentrated to *ca.* 20 c.c. and allowed to crystallize for 24 hours at room temperature. The colourless crystals (0.18 g.) so obtained gave no colour reaction with aqueous ferric chloride and seemed to be pure; m.p. 144.5°. They were analysed, since preliminary experiments had shown that the substance is decomposed by repeated recrystallization. (Found: C, 60.5; H, 4.6. Equiv. 173. $C_{14}H_{14}O_6$ requires: C, 60.0; H, 4.5 per cent. Equiv. 180.)

SUMMARY

The antimitotic properties of maleic acid, citraconic acid, fumaric acid and mesaconic acid have been examined in cultures of chick fibroblasts *in vitro*. Maleic acid proved to be a strong antimitotic.

The connection of this group of substances with the 1:4–quinones has been discussed.

In the hydroquinone series 1:4–naphthohydroquinone diphosphate has been prepared and its antimitotic properties have been examined. It was found to be 1,000 times as active as 2–methyl–1:4–naphthohydroquinone diphosphate. 1:4–Napthoquinone mono-hydrogen succinate was completely inactive.

One of us (E. F.) is indebted to Eli Lilly and Company for financial support.

REFERENCES

Barber, H. N., and Callan, H. G. (1943). *Proc. Roy. Soc.*, **131B**, 258.

Broderson, H. (1943). *Strahlentherapie*, **73**, 196.

Brunschwig, A., Arnold, J., and Edgcomb, J. (1946). *Cancer Res.*, **6**, 560.

Cook, J. W., Graham, W., and (in part) Cohen, A., Lapsley, R. W., and (the late) Lawrence, C. A. (1944). *J. chem. Soc.*, 322.

Dewar, M. J. S. (1945). *Nature, Lond.*, **155**, 141.

Dustin, A. P. (1934). *Bull. Acad. Méd. Belg.*, **17**, 487.

Dustin, A. P. (1939). *Arch. exp. Zellforsch.*, **22**, 395.

v. Euler, H., and v. Hevesy, G. (1942). *K. Danske vider skal. Biol. Med.*, **17**, Nov. 8.

Huber, W. (1947). *Rev. Suisse Zool.*, **54**, 63.

Kopac, M. J. (1945). *N. Y. Acad. Sci.*, Ser. II, **8**, 5.

Lehmann, F. E. (1942). *Verh. Ver. Schweiz. Physiol.*, June.

Lehmann, F. E. (1945). *Rev. Suisse Zool.*, **52**, 343.

Lehmann, F. E. (1946). *Rev. Suisse Zool.*, **53**, 475.

Lettré, H. (1946). *Naturwissenschaften*, **33**, 75.

Lettré, H., and Albrecht, M. (1941). *Hoppe-Seyl. Z.*, **271**, 200.

Lettré, H., and Albrecht, M. (1943). *Hoppe-Seyl. Z.*, **278**, 201.

Lettré, H., Telschow, H., and Kruger, I. (1946). Unpublished, quoted from *Naturwissenschaften*, **33**, 75.

Ludford, R. J. (1936). *Arch. exp. Zellforsch.*, **18**, 411.

Lüscher, M. (1946). *Helv. physiol. pharmacol. Acta*, **4**, 465.

Meier, R., and Allgöwer, M. (1945). *Experientia*, **1**, 57.

Meier, R., and Schär, B. (1947). *Experientia*, **3**, 358.

Mitchell, J. S. (1942). *Brit. J. exp. Path.*, **23**, 285, 296, 309.

Mitchell, J. S. (1946). *Schweiz. med. Wschr.*, **76**, 883.

Mitchell, J. S., and Simon-Reuss, I. (1947). *Nature, Lond.*, **160**, 98.

v. Möllendorff, W. (1938). *Z. Zellforsch.*, **28**, 512.

v. Möllendorff, W. (1939). *Z. Zellforsch.*, **29**, 706.

Pomerat, C. M., and Willmer, E. N. (1939). *J. exp. Biol.*, **16**, 246.

Töndury, G. (1941). *Verh. Schweiz. naturf. Ges.*, 167.